AD_____

GRANT NUMBER DAMD17-97-1-7098

TITLE: Epitope Specific T-Cell Immunity to Breast Cancer

PRINCIPAL INVESTIGATOR: Constantin G. Ioannides, Ph.D.

CONTRACTING ORGANIZATION: M.D. Anderson Cancer Center
Houston, Texas 77030

REPORT DATE: August 1998

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 1998	Annual (1 Aug 97 - 31 Jul 98)

4. TITLE AND SUBTITLE

Epitope Specific T-Cell Immunity to Breast Cancer

5. FUNDING NUMBERS

DAMD17-97-1-7098

6. AUTHOR(S)

Ioannides, Constantin G., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

M.D. Anderson Cancer Center
Houston, Texas 77030

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

We analyzed the ability of T cells from PBMC of breast cancer patients to recognize HER-2/neu (HER-2) peptides. We found that 13/18 patients responded by proliferation to at least one of the HER-2 peptides tested. Of these peptides, one designated G89 (HER-2:777-789) was recognized by T cells from 10 patients. 7/9 responding patients were HLA-DR4^{+}, suggesting that this peptide is recognized preferentially in association with HLA-DR4. Analysis of the specificity and restriction of the cytokine responses by G89-stimulated T cells revealed that these cells secreted significantly higher levels of IFN-γ than IL-4 and IL-10 suggesting priming for a T helper 1 (Th1) response. The same pattern of responses was observed to the intracellular domain (ICD) of HER-2 suggesting that G89-stimulated T cells recognized epitopes of the HER-2 protein in association with HLA-DR4. Since HLA-DR4 is present in 25% of the humans, characterization of MHC-class II restricted epitopes inducing Th1 responses may provide a basis for the development of multivalent HER-2 based vaccines against breast and ovarian cancer.

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
53

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

C.G.1 ✓ Where copyrighted material is quoted, permission has been obtained to use such material.

C.G.2 ✓ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

C.G.3 ✓ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

C.G.4 N/A In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

C.G.5 ✓ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

C.G.6 ✓ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

C.G.7 ✓ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

C.G.8 ✓ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

C. G. Ioannides

CONSTANTIN G. IOANNIDES 8/20/98

PI - Signature Date

TABLE OF CONTENTS

Abbreviations used: Cytotoxic T lymphocytes, CTL; Peripheral blood mononuclear cells, PBMC; Antigen, Ag; Polymerase chain reaction, PCR; T cell receptor, TCR; HER-2/neu proto-oncogene, HER-2; stimulation index, S.I.; standard deviation, SD; tetanus toxoid, TT; influenza hemaglutinin, HA; wild-type, w.t.; HER-2 intracellular domain, ICD; HER-2 extracellular domain, ECD, lymph node, LN.

INTRODUCTION

The overall objective of these studies is to develop novel therapeutic approaches to breast cancer by understanding the requirements for successful induction of anti-tumor responses using newly defined T cell epitopes. The use of peptides mapping immunogenic epitopes can overcome limitations in the use of whole self-protein or biased involvement of Th2 cells. Our recent studies identified an immunodominant CTL CTL epitope on HER-2(1). Despite the presence of specifically reactive CTL with epitopes on autologous tumor, the disease progresses suggesting that this response is either too weak or suppressed. An approach to this problem is to use the TH1 subset of $CD4^+$ cells to induce and amplify a CTL response to tumor because it was implicated in the regulation of anti-self responses, and re-activation of pre-existent CTL response.

Thus, a novel approach to induction and amplification of specific tumor effectors in breast cancer is to re-activate tumor (self) Ag specific Th1 cells. The relevance of Th1 and CTL recognizing defined epitopes is four-fold: **(a)** Th1 cells recognizing epitopes on tumor Ag provide the immunological memory for activation of a CTL response when the Ag is presented by the tumor or shed. Since the same MHC class II molecule associates with different class I, the Th1 response will be effective for multiple CTL epitopes expressed by distinct HLA,A,B,C types (2,3). **(b)** The ability of Th peptides to induce and amplify a CTL response to the newly defined epitope E75(HER-2: 369-377) will establish the right timing for a therapeutic response; **(c)** Th peptides of optimized sequence allow activation of anti-tumor responses; Conversely other sequence changes inhibit a chain of deleterious responses; **(d)** the use of defined CTL and Th epitopes allow rational design of specific cancer vaccines.

During our previous studies we identified several HER-2 peptides which induced proliferative responses in healthy donors and ovarian cancer patients (4). These ovarian cancer patients had advanced disease. We wanted to examine the significance of these epitopes in breast cancer patients. We focussed on a specific group of patients, breast cancer patients that were clinically free of disease at the time of study. The peptides found to be active in inducing proliferation in ovarian cancer patients and healthy donors were tested together with analogs

containing sequence motifs for binding to HLA -DR4. HLA–DR4 was chosen because of the recently reported association between HLA-DR4 and favorable prognosis in breast cancer.

This study aimed to identify whether cells responding to such HER-2 peptides, by proliferation, exist in the peripheral blood of these patients, and if they exist, then they can be expanded and their cytokine pattern can be characterized. We were particularly interested in identifying peptides that induced TH1 responses demonstrated by significantly higher levels of IFN-γ secretion than of IL-4. *Additional objectives of these studies were (a) to determine whether the responses to these peptides are affected by the presence of tumor, i.e., responses to which peptides are suppressed and (b) whether we can induce determinant spreading, i.e., expansion of responsiveness to other peptides by primary stimulation with the helper peptide.*

The objectives of the determinant spreading study were to determine whether: (1) tolerance of cellular responses to a tumor Ag can be overcome in healthy donors; (2) this leads to activation of responses to cryptic epitopes; and (3) a Th1 response can be elicited by consecutive or alternative stimulation with the same or different peptides. We found that primary in vitro stimulation with some HER-2 peptides, leads to spreading of proliferative responses to other HER-2 peptides. Spreading of these responses is paralleled by secretion of significantly higher amounts of IFN- than IL-4. These results suggest that a sequential determinant spreading can be achieved by stimulation with HER-2 peptides. This may provide a basis for prediction of determinant spreading for therapeutic intervention in developing anti-tumor vaccines.

MATERIALS AND METHODS

Subjects. Peripheral blood mononuclear cells (PBMC) were obtained from 18 breast cancer patients and 6 healthy volunteers. All patients, with one exception, were clinically free of tumor at the time of study. Eleven patients had pathologis stage I disease, 5 had stage II, and 1 stage 3. One patient (No. 16) had no primary tumor yet was classified as having breast cancer. HER-2 staining for the autologous breast tumors was performed by immunocytochemistry. For determinant spreading studies, peripheral blood was collected from two healthy donors designated as Donor A (HLA-A2, 23, B7.48, DR7, 11, DQ2.6) and Donor B (HLA-A11, 68, B51, 67, DR13, 14, DQ5, 6) and from an ovarian cancer patient, designated as patient A (HLA-A24, 28, B35, W6, 70, Cw3,4, DR11, 14, DQ5).

HLA class II molecular oligotyping. Genomic DNA was extracted from PBMC as described (5); This DNA served as the substrate for amplification of a polymorphic locus-specific fragment of the HLA class II gene by polymerase chain reaction (PCR). For the *-DQB1* and *-DRB* loci, the flanking primers used were:
DRB-AMP-A: 5'CCCCACAGCACGTTTCTTG;
DRB-AMP-B: 5'CCGCTGCACTGTGAAGCTCT;
DQB-AMP-A:5'CATGTGCTACTTCACCAACGG; and
DQB-AMP-B: 5'CTGGTAGTTGTGTCTGCACAC.

Because of the large number of *HLA-DRB* alleles and the numerous shared sequences between different alleles, *HLA-DRB* typing was carried out in a stepwise manner. First, group-specific *HLA-DR* typing was performed using the primers DR-AMP-A and DR-AMP-B. Oligonucleotide typing of this PCR-amplified DNA allowed discrimination of *HLA-DR1, -DR2, -DR3/6,-DR4, -DR5 (-DRw11), -DR7, -DR8/12, -DR9, -DR10, -DR52a, -DR52b/c*, and *-Drw53*. Since there are numerous variants of *HLA-DR1, -DR2, -DR4, -DR5 (-DRw11), -DR6, -DR8/12* and *-DR52b/c,* further discrimination of these subtypes required a second PCR using group-specific primers plus DRB-AMP-B. They include DRB-AMP-1 for the *HLA-DR1* group, DRB1-AMP-2 or DRB5-AMP-2 for the *HLA-DR2* group, DRB-AMP-3 for the *HLA-DR3, -DR5, -DR6,*

-DR8, -DR12 group, DRB-AMP-4 for the *HLA-DR4* group, and DRB-AMP-52 for the *HLA-DRB3* genes of the *HLA-DRw52* group. The sequences of the primers were as follows:
DRB-AMP-1; 5'TTCTTGTGGCAGCTTAAGTT;
DRB1-AMP-2; 5'TTCCTGTGGCAGCCTAAGAGG;
DRB5-AMP-2; 5'CACGTTTCTTGCAGCAGGA; and
DRB-AMP-4; 5'GTTTCTTGGAGCAGGTTAAC;
HLA-DRw52-associated *-DRB1* genes (*HLA-DR3, -DR5, -DR6, -DR8*, and *-DR12*): DRB-AMP-3; 5'CACGTTTCTTGGAGTACTCTAC; *HLA-DRw52*; DRB-AMP-52; 5'CCCAGCACGTTTCTTGGAGCT

PCR products were analyzed by electrophoresis. The amplified DNA was blotted to Hybond N+ membranes (Amersham, Arlington Heights IL) hybridized with γ^{32}P-ATP-labeled allele-sequence specific oligonucleotide probes (SSO). *HLA-DQB1* alleles were determined by hybridization with probes corresponding to variable sequences around positions 23, 26, 37, 45, 49, 57 and 70 of the *HLA-DQB1* outermost domain. "Broad" *HLA-DR* groups [*HLA-DR1, -DR2, -DR3/6, -DR4, -DR5 (11), -DR12, -DR7, -DR8, -DR9, -DR10, -DRB3*0101, -DRB3*0201-*0301, -DRB4*0101 (-DR53)*] were determined by hybridization with oligonucleotide probes corresponding to variable sequences around positions 10, 28 and 37 of the *HLA-DRB1* outermost domain. Subtypes of *HLA-DR1, -DR2, -DR3/5/6/8/12, -DR4* and *-DRw52* were determined by hybridization of the respective group-amplified DNA to oligonucleotides corresponding to variable sequences around positions 28, 37, 57, 70 and 86 of the *HLA-DRB1* outermost domain.

HER-2 peptide selection. Peptides tested were selected if they contained the T cell sites in HER-2 predicted by the computer program ANT.FIND.M, the general binding motif for human class MHC-II antigens, and the anchors for a number of MHC-class II antigens: HLA-DR1, -DR3, -DR4, -DR11, and -DQ7 (6, 7), the sum of whose allelic frequencies covers between 75 - 100% of the Americans. The general peptide binding motif for various human MHC-class II molecules consists of a position 1 (P1) anchor, i.e. an aromatic or large aliphatic residue in the first 3 - 5 amino acids close to the N-terminus and other major but less essential anchors at P4, P5, P7, and P9 counting from the P1 anchor (7, 8). Since many peptides are capable of binding to many different MHC-class II molecules, because their sequences contain overlapping binding

motifs for MHC-class II molecules (7, 8), each of the peptides synthesized contained at least two of three anchors for each HLA-DR antigen, and the main P1 anchors for most class II alleles **(Table II)**. In peptides G88, G89, and G90, positions P3 and P4 are occupied by hydrophobic, aromatic followed by aliphatic residues in that order to facilitate peptide binding in different frames.

The binding motifs of synthetic peptides may differ from those of natural ligands because the latter incorporate processing constraints in addition to binding requirements. Thus peptides were synthesized by following, when possible, the common motifs for all MHC-class II molecules defined by pool sequencing of naturally processed peptides. The sequences of peptides used in this study are as follows: F12 (449-465) GIS*W*LGLRSRELGSGL; G88 (450-463): ISWLGLRSRELGS; F7 (776-789) GSP*Y*VSRLLGICL; G89 (777-790): SPYVSRLLGICL; F13 (884-899) VPIKWMALESILRRRF; G90 (886-898): IKWMALESILRRR. In F7, F13 and F14, the sequence was extended to include Pro (P) N-terminal to either the Tyr (Y), (the P1 anchor for HLA-DR1, DR3, DR4, and DQ7), or Trp (W) (reportedly by the P1 anchor for HLA-DR4 and DR11). For comparative studies of the responses associated with HLA-DR4, 13-mer analogs of F12, F7 and F13 G88, G89 and G90 respectively were selected, using the anchor alignment matching the standard HLA-DR4/DR1 helper epitope, influenza HA peptide (HA:307-319). The predicted binding affinities of these peptides for HLA-DR4 (as IC50) according to Rothbard's algorithm were as follows: HA, 35 nM, G88, 180 nM, G89, 987 nM, and G90, 219 nM (9). Peptides 13-16 residues long **(Table I)** were prepared by the Synthetic Antigen Laboratory of M.D. Anderson Cancer Center, using a solid phase method as previously described (10). Their identity was determined by amino acid analysis. Their purity was 93 - 97% as determined by HPLC. Peptides were dissolved in PBS, aliquoted at 2 mg/ml and stored frozen at -20°C until use. The codes used to identify these HER-2 peptides in this paper were assigned by the Synthetic Antigen Laboratory.

Recombinant intracellular HER-2 domain. The HER-2/neu intracellular domain (ICD: K676-V1255) was cloned by PCR from c-erbB-2 cDNA (provided by Dr. Jacalyn Pierce, NCI). The ICD was expressed in E.coli using a pET vector with an amino terminal His tag. The expression of ICD protein was induced with IPTG and E.coli pellets harvested after an additional

4 hours. Recombinant ICD was purified from inclusion bodies by a combination of Ni^{++} affinity chromatography, size exclusion and ion exchange chromatography. The purified ICD was greater than 95% pure as judged by PAGE and Western analyses (data not shown).

Stimulation and propagation of T cells. Freshly harvested PBMC from breast cancer patients and healthy volunteers were isolated by Ficoll/Hypaque centrifugation. $CD4^+$ and $CD8^+$ T cells were isolated from the plastic using non-adherent fractions. Cells were cultured at 1 x 10^6 cells/ml in RPMI-1640 (Gibco, Grand Island, NY) medium with 10% pooled human AB serum and antibiotics in 2 ml in each well of a 24-well plate (complete RPMI medium). HER-2 peptides were added to selected wells at a final concentration of 25 μg/ml. In other wells, PBMC were stimulated with 5 ug/ml of tetanus toxoid (TT), 25 ug/ml HA peptide, PHA at a final concentration of 1:100 or medium alone. After 6 days of stimulation with each peptide, cultures were expanded with IL-2 (Cetus) at 20 U/ml for the following week (17, 31). To induce antigen specific T cells, the cells were then "rested" for 3 - 4 days by culture in the absence of IL-2. Then, the cells were stimulated at a 1:1 (stimulator:responder ratio) with irradiated (10.000 Rad) PBMC and pulsed with individual peptides for at least 90 min at 37°C before addition to the cultures as described (4). Control cultures were stimulated with the same number of PBMC in the absence of peptides. For expansion, four to five days later 20 U/ml IL-2 was added to the cultures for seven additional days. Surface antigen expression was determined by FACS analysis using a FACSscan (Beckton-Dickinson, Sunnyvale, CA) with a log amplifier. CD3, CD4 and CD8 antigen expression on T cell cultures was determined by immunofluorescence with corresponding mAb FITC-conjugated (Beckton Dickinson).

Proliferation assays. For proliferation assays 100 μl aliquots were removed from each well of the 24-well plate of primary cultures after 4-6 days as described (11). Tetraplicate samples were cultured in a 96 well plate with 1 μCi [^{3}H]-Tdr in a final volume of 200 μl. The cells were harvested 16 h later, and the radioactivity counted in a Beckman LS3501 liquid scintillation counter (11). A positive proliferative response was defined as positive when differences in cpm values between cultures that received peptides compared with cultures which did not receive peptides were significant by the unpaired Student's t-test ($p < 0.05$). Stimulation

indexes (S.I.) represented the ratio between the mean c.p.m. of the cultures stimulated with peptide, and the mean c.p.m. of the cultures that have not been stimulated with peptide (N.P.).

Cytokine production. The ability of PBMC to secrete antigen-induced IFN-γ, IL–4, and IL-10 was determined by culturing the PBMC with the corresponding peptides. Supernatants were collected at different times and stored frozen at -20°C. The cytokine concentrations were measured by double sandwich-ELISA using the corresponding kits provided by BioSource International (Camariyo, CA). The cytokine assays were calibrated with human recombinant IFN-γ, IL-4, and IL-10 to detect each cytokine in the range of 15-1000 pg/ml. The following homozygous B cell lines were obtained from the American Society for Histocompatibility and Immunogenetics Repository (Baltimore, MD) and used as APC for cytokine secretion: E418 (DRB1*1502, DRB5*0102, DQA1*0102, DQA1*0103, DRB1*0601) and WT51 (DRB1*0401, DRB4*0101, DQA1*0301, DQB1*0302).

Statistical Methods. Differences in proliferative responses were analyzed using Student's t-test for unpaired samples. Differences in frequency for class II alleles were assessed using the Cochran Q test (32).

RESULTS

1) *Characterization of antigenic HER-2 peptides inducing proliferative and IFN-γ responses of PBMC from breast cancer patients.* To identify HER-2 peptides that induce proliferation of peripheral blood mononuclear cells from breast cancer patients, we focussed on HER-2 peptides that have been demonstrated to induce proliferation in healthy donors and ovarian cancer patients (1) (Fisk, et., Anticancer Res. 17: 45-54, 1997 Appendix A attached). These preliminary studies showed that three HER-2 peptides corresponding to positions 396-406(D122) 474-487 (F12), 777-789 (F7) and 886-899 (F13) were able to induce proliferation in the majority (60 – 70%) of the healthy donors tested. Of these peptides, F7, and F13 were also able to induce T cell proliferative responses more frequently (20 – 27%) in a large group

of ovarian cancer patients (n=25). This suggested that responses to such peptides are maintained even during disease progression.

Since both healthy donors and ovarian cancer patients have not been HLA-typed it was important to establish whether such responses are preferentially observed in association with a certain HLA-type. Since cancer vaccines development and therapeutics, it is more likely to be effective in patients that are in early stages of disease, or in patients that are free of disease after surgery and chemotherapy, we decided to focus these studies on breast cancer patients that are disease free. Since HLA-DR4 was reported to be associated with a favorable prognosis in breast cancer, we decided to include a significant group of HLA-DR4^{+} patients. To ensure that the MHC-II molecular type is known, all patients were typed for both HLA-DR and HLA-DQ. This was possible by a collaboration with Drs. William E. Thompson and Jeffrey E. Lee from the Department of Surgical Oncology. As described in the Material and Methods Section, this molecular approach allows precise characterization of the Class II-MHC phenotype from 24 distinct samples (18 breast cancer patients and six healthy donors). The results of this typing are shown in the attached publication (Tuttle, et.al, Clinical Cancer Research, 4:2015-2024, 1998, see attached), **Table II**.

To identify Her-2 peptides that induced responses in these breast cancer patients, we tested for proliferation induction peptides F7, F13, and F14 in the same experiment. We also generated analogs of these peptides based on the sequence motifs characteristic for peptide binding to HLA-DR4, following all the general motifs for HLA-DR4, (also found in the sequence of the standard influenza virus hemagglutinin (HA) peptide epitope HA:307-319. It is important to note that the sequence of one of these peptides: G89 (HER-2, 777-789), was identical with that of F7 with only two aminoacids shifted, one N terminal and one C terminal.

To establish whether these peptides can activate T cells, we determined the proliferative responses to these peptides over a six day interval. Since the patients immunoreactivity is low, we determined the proliferative responses for three consecutive days (Days 4, 5, and 6) after stimulation. We considered a positive response when the cpm in each of the

quadruplicate cultures stimulated with peptide was higher than each of the quadruplicate control cultures on two consecutive days. This approach was introduced to identify responses without arbitrary cut-offs by setting as threshold a specific magnitude of the responses.

In, likely, the most extensive study performed on the responsiveness of breast cancer patients to HER-2 peptides, we found that 12 of 18 (66%) of the breast cancer patients responded to at least one HER-2 peptide. Of these patients ten (55%) responded to the peptide G89. Of these ten responders to the peptide G89, five were HLA-DR4^{+}. Therefore, the results show a potential association (7 of 9, 77%) of responses to G89 with HLA-DR4 **(Table II)**.

In addition, three HLA-DR4^{-} patients responded with significant proliferation to F13. Responses to F13 and its shorter analog G90 were observed in other patients which shared HLA-DR7. These results also show that HER-2 peptides can induce proliferative responses even in patients which express low levels of HER-2 on their primary tumors, supporting that cells of these specificies exist.

To address the significance of these findings, we characterized the proliferative and cytokine responses to G89 in a breast cancer patient and a healthy HLA-DR4^{+} donor in parallel. We found that G89 specific T cells can be expanded even from the PBMC of the healthy donor, and they specifically recognized peptide G89. Furthermore, these cells, both from the patients and from the healthy donor secreted high and specific levels of IFN-γ in response to G89. In contrast, they secreted low and insignificant levels of interleukin-4 (IL-4) and interleukin-10 (IL-10). These responses appeared to be peptide induced and specific for the peptide G89, because stimulation in parallel in the same conditions with the peptide G90 of PBMC of the healthy donor lead to cells that secreted high levels of both IFN-γ and IL-10, suggesting that G90 may be a cross reactive epitope. (Tuttle, et.al. Clinical Cancer Research 4, 2015-2024, see attached and **Figure 1**. (Appendix).

2) *Activation of proliferative responses of T cells from breast cancer patients lymph nodes by HER-2 peptides.*

Breast Cancer spreads initially through the lymph nodes (LN). To determine how the presence and absence of tumor cells affect the responses to G89, we identified an HLA-DR4 patient, from whom three freshly resected lymph nodes (sections of nodes) were available. Tumor was present in one node, whereas the two other nodes, proximal and distal from the first lymph node were free of tumor. To determine the effects of the tumor presence on the responses to HER-2 peptides, the three lymph nodes were processed simultaneously, and the lymphocytes from each LN were cultured with either no peptide (NP), as negative control, or tetanous toxoid (TT) as positive control, F13 as specifity control and the peptides F7 and G89. It should be noted that F7 and G89 differ only in the N and C terminal residues. We found that by comparing the stimulation indexes, responses to G89 were lower (1.9vs. 3.1, vs. 5.4) in the LN with tumor than in the distal LN, suggesting that G89 reacting cells may be preferentially affected by the tumor or tumor secreted factors. The ratio between S.I in the distal LN and the LN with the tumor was: (5.4:1.9=2.84). In contrast, responses to the F7 were significantly less affected by the tumor (S.I = 3.8, vs. 4.5 vs. 5.4). The ratio between S.I. (F7) in the distal and Tumor LN was 4,5:3.8=1.2 **(Figure 2)**.

To determine the significance of these proliferative responses with respect to cytokine production we determined the levels of IFN.γ and IL-4 secreted by LN cells pre-primed with HER-2, as well as the positive control tetanous toxoid. To assure that the results are representative of the frequency of the producers in the population, equal numbers of peptide stimulated live cells were used in each experiment . Thus, the results were not affected by the different rates of proliferation previously identified. To determine the cytokine secreting potential all cells were stimulated with TPA + OKT3 mAC as we described. The levels of IFN-γ and IL-4 are shown in **Figure 3A, B.**

The results show that stimulation with HER-2 peptides and tetanous toxoid has different effects in inducing IFN-γ and IL-4. The ratio IFN-γ:IL-4 in the unstimulated and F7 stimulated cells was similar suggesting that although F7 induced higher proliferation did not significantly affect the balance or the cytokine secretion potential of TH1/TH2 subsets. In contrast, peptides F13 and G89 induced higher IFN-γ:IL-4 ratios which were due to decreased IL-4 levels. The ability of stimulating Ag to induce different levels of cytokine is

also illustrated by the IL-4 levels in response to TT. Thus, our results show that the IFN-γ stimulation effect by G89 is sequence specific since it is not seen with the overlapping peptide F7. The results also confirm the ability of G89 to induce IFN-γ compared with the other HER-2 peptides.

Induction of determinant spreading by the HER-2 peptides

We first tested the ability of PBMC, from donor A, to respond to peptides G88, G89, G90 and their corresponding longer analogs F12, F7 and F13. The MHC-class II phenotype of this donor is HLA-DR7/11, HLA-DQ2, 6. This donor was chosen because we have seen that some breast cancer patients respond to both F7(G89) and F13(G90). These patients expressed HLA-DR7/11. We wanted to address the question whether stimulation with any of these peptides, can prime the T cells to respond to the same or other peptides on secondary stimulation. The proliferative responses were determined after both five and six days after the primary stimulation. This was made to assure that if responses are observed they are stable and consistent over a 48-hour period. The results are shown in **Figure 4A, B**. Neither peptide induced a significant proliferative response by Donor A PBMC. The immunodominance of peptide F12 on day 5 was short lived and not detected on day 6 (Not shown). Therefore, these HER-2 peptides behaved as cryptic determinants.

To address the possibility that the anti-HER-2 peptide responses and repertoire diversifies in peptide primed PBMC, we examined the specificity of the HER-2 responses two weeks after the primary stimulation with each peptide. We focused on F7, F12, and F13. To ensure that all the repertoires have the opportunity to expand, all cultures including the control PBMC which have not been stimulated with peptides received 20U/ml IL-2 for 4 days. Afterwards IL-2 was gradually removed over five additional days, and the responders were rested by being cultured in media without IL-2 for two additional days. Afterwards, the cells were restimulated with autologus plastic adherent PBMC as APC pulsed with each of the peptides F7, F12, and F13 or control (no peptide). The results of proliferative responses were determined again on days 5 and 6 **(Figure 4C, D respectively)**.

Strong proliferative response were recalled by peptides F7 and F13, compared with the cultures that were not subjected to primary stimulation with peptides. Of interest, determinant spreading could be observed with both F7 and F13, but F13 appeared dominant **(Figure 4C)**. A similar pattern of responses was observed by determining the levels of proliferation six days after restimulation although responses to both F7 and F13 decreased as indicated by the SI. F12 could recall only weak responses in the cultures previously stimulated with F13 but at lesser extent in the cultures stimulated with F3 and F12. These results show that unlike F7 and F13 there is a higher degree of self-tolerance to F12 in this donor.

The observed priming to formerly "cryptic-self" and the dominance of F7 suggest a cytokine mediated reaction that may assist the induction of a second and third round of priming to cryptic determinants of HER-2. To address this question we determined the levels of IFNγ and IL-4 in F7F7, F7F13, F13F7, and F13F13 cultures **(Figure 5)**. We found significantly higher levels of IFNγ in the cultures initiated with F7 and recalled with F7 than recalled with F13. In contrast, in the cultures initiated with F13 the recall with either F7 or with F12 but not with F13 induced higher levels of IFNγ, supporting an increased correlation between the and IFNγ secretion. In all these cultures the levels of IL-4 were significantly lower than the levels of IFNγ.

To address the question whether epitopes F7, F12, and F13 are operational in other MHC-class II restriction systems we tested their ability to stimulate and recall responses in the Donor B. The HLA-DR phenotype of donor B, DR13,14 is entirely different from Donor A. The results are shown in **Figure 6A.** F7, F12 and F13 failed to recall proliferating responses of PBMC from Donor B. This suggests that the ability of certain HER-2 epitopes to induce auto-immune responses may be evident only in association with certain MHC-types. To determine whether the stimulated cells secreted cytokines, we determined the levels of IFN-γ and IL-4 in the same conditions as for the Donor A. The results are shown in **Figure B**. Although in both donors the levels of IFN-γ detected at the primary stimulation were minimal (data not shown), the IFN-γ levels secreted by Donor B in response to F7 and F13 stimulation in any order were significantly lower than the levels determined from Donor A. This suggests that stimulation

induced by these HER-2 peptides in Donor B may be both minimal and not sufficient for determinant spreading and repertoire expansion.

Similar results as with Donor A were obtained with an ovarian cancer patient of similar MHC-Class II Type. We used in this study an ovarian cancer patient because she was available for repeated blood withdrawal (Proceedings AACR, 1998, A574, Attached). These results indicate that determinant spreading can be induced not only in healthy donors but also in cancer patients.

CONCLUSIONS

(1) We found that PBMC from primary breast cancer patients respond by proliferation in vitro to a number of HER-2 peptides. The responding population consists of $CD4^+$ cells, as suggested by the ability of the responding cells to secrete IFN-γ in response to these peptides when presented by MHC-class II. The frequency of the responses was higher for G89 (56%) than for the other peptides tested, suggesting that G89 may represent an immunodominant epitope in the group analyzed. Of interest, the responses to G89 appeared to associate more frequently with the presence of HLA-DR4 (in 7/9 cases) suggesting that HLA-DR4 may be the presenting element.

(2) F7 and G89 are equal in length but differ by one residue at their N- and C-terminal ends. This suggests that the epitope formed by G89 *in vitro* when used at a concentration of ~10 μM is specifically recognized. The frequency of responses appeared not to be related to the binding affinity of these peptides to DR4. The predicted binding affinity of G89 to HLA-DR4 was significantly lower than that of peptides G88 and G89 of the same length. T-cell cultures primed with peptide responded at restimulation by secreting more IFN-γ than IL-4 and IL-10, suggesting the preferential activation of a Th1 response. This response was apparently not directed to a cryptic HER-2 epitope since peptide-primed cells recognized the ICD. Although the results of this study do not rule out the presence of cross-reactive epitopes with G89 in the ICD, it should be mentioned that a T cell line induced by stimulation with G90 (located in the ICD, at amino acids 886-898) showed cross-reactive recognition with the ECD epitope G88 (450-462). The IFN-γ response to ICD of G89-primed T cells suggests that HLA-DR4 may be the presenting element for a naturally processed epitope similar in structure to G89. Since HLA-DR4 is expressed in approximately 25% of humans, this epitope may be an important peptide for activation and regulation of T cell differentiation toward a Th1 response. It may also be beneficial for CTL activation and expansion.

(3) This pattern of responses raised the question whether the G89-induced Th1 response plays a protective role, during tumor spread or if it is down-regulated by Th2 cytokines

subsequent to recognition of other peptides after HER-2 overexpression. Ongoing studies in our laboratory are attempting to clarify these points. Characterization of epitopes that regulate Th1 responses, which can in turn control the spread of Th1/Th2 responses by other self-peptides, may have important implications not only for CTL induction but also for understanding the regulation of human tumor immunity. In this direction, we found that T cells from lymph nodes infiltrated with tumor show decreased proliferation to G89 and F13 compared with F7. The ability of LN cells to respond to G89 increased with the distance from tumor. This raised the possibility that G89 specific cells may be suppressed or energized by the tumor and may play a role in tumor surveillance.

(4) During ongoing studies, we have developed as APC, dendritic cell (DC) derived from plastic adherent PBMC. These APC are currently used to develop $CD4^+$ lives from breast cancer patients recognizing the epitope mapped by G89. In preliminary studies (which are not included) in this report, we found that stimulation of T cells with the CTC epitope ETS plus the helper epitope G89 induce higher levels of IFN-γ production than each peptide above suggesting a synergy between these two epitopes. Furthermore, we observed that G89 can substitute for IL-12 regarding IFN-γ induction. Since a major problem related to development of tumor specific CTL relates to our lack of ability to induce massive CTL expansions. We plan to investigate the ability of G89 to upregulate $CD4^+$ on DC, the ability of IL-15 to protect $CD4^+$ and $CD8^+$ cells from apoptosis.

REFERENCES

1. Fisk, B., Blevins, T. L., Wharton, J. T., and Ioannides C. G. 1995. Identification of an immunodominant peptide of HER-2/neu-proto-oncogene recognized by ovarian tumor specific CTL lines. *J.Exp. Med. 181*:2709-2717.

2. Lee, JE, Reveille, JD, Ross, MI, Platsoucas, CD. 1994. *HLA-DQB1*0301* association with increased melanoma risk. Int J Cancer *59*:510-513.

3. Sinigaglia, F., and Hammer, J. 1995. Motifs and supermotifs for MHC class II binding peptides. *J. Exp. Med. 181*:449-451.

4. Fisk, B., Hudson, J. M., Kavanagh, J., Wharton, J.T., Murray, J.LO., Ioannides, C.G. and Kudelka, A.P. 1997. Existent proliferative responses of peripheral blood mononuclear cells from healthy donors and ovarian cancer patients to HER-2 peptides. *Anticancer Res. 17*:45-54.

5. Lee, J.E., Lowy, A,M., Thompson, WA, Lu, M, Bell, B, Skibber, JM, Evans, DB, Curley, SA, Mansfield, PF, Reveille, JD. Association of gastric adenocarcinoma with the human leukocyte antigen class II gene *DQB1*0301*. *Gastroenterology 111*:426-432.

6. Kropshofer, H., Max, H., Muller, C.A., Hesse, F., Stevanovic, S., Jung, G., and Kalbacher, H. 1992. Self-peptide released from class II HLA-DR1 exhibits a hydrophobic two-residue contact motif. *J.Exp.Med. 175*:1799-1803.

7. Wucherpfennig, K.W., and Strominger, J.L. 1995. Selective binding of self peptides to disease-associated major histocompatibility complex (MHC) molecules: a mechanism for MHC-linked susceptibility to human autoimmune diseases. *J.Exp.Med. 181*:1597-1601.

8. Hammer, J., Valsasnini, P., Tolba, K., Bolin, D., Higelin, J., Takacs, B., and Sinigaglia, F. 1993. Promiscuous and allele-specific anchors in HLA-DR binding peptides. *Cell. 74*:197-203.

9. Marshall, K. W., Wilson, K. J., Liang, J. Woods, A., Zaller, D., and Rothbard, J. B. 1995. Prediction of peptide affinity to HLA-DRB1*0401. *J. Immunol. 154*:5927-5933.

10. Ioannides, C. G., Fisk, B., Fan, D., Biddison, W. A., Wharton, J. T., and O'Brian, C. A. 1993. Cytotoxic T cells isolated from ovarian malignant ascites recognize a peptides derived from the HER-2/neu proto-oncogene. *Cell. Immunol. 151*:225-234.

11. Disis, M.L., Calenoff, E., McLaughlin, G., Murphy, A.E., Chen, W., Groner, B., Jeschke, M., Lydon, N., McGlynn, E., Livingston, R.B., Moe, R., Cheever, M.A. 1994. Existent T-cell and antibody immunity to HER-2/neu protein in patients with breast cancer. *Cancer Res 54*:16-20.

APPENDICES

Figure 1. Cytokine secretion by G89R, G89L, and G90L T cells (5 x 10^4 cells each) in response to G89 and G90 respectively pulsed on autologous irradiated PBMC from donor 3 (1 x 10^5). G88 was used as control. Between 70-80% of cells had $CD4^+$ phenotype. Cytokine secretion by G89R, G89L and G90L was measured in the same experiment as described in the Materials and Methods. IFN-γ, IL-4, IL-10. *Indicates that levels of this cytokine were below the sensitivity of the assay (2 μg/ml).

Figure 2. HER-2 peptide induced proliferation of LN cells from a breast cancer patient. (LN3) tumor indicate that breast tumor was present at the time of resection. LN1 was macroscopically tumor free and closer to the LN3 than LN2 (designated distal). SI indexes were calculated as described in the Materials and Methods. LN cells were stimulated with epitope 10 μg/mlof each peptide on 5/μg/ml of TT. 3H-TdR was determined on day 4 from tetraplicate cultures.

Figure 3A, B. IFN-γ (A) and IL-4(B) secretion by LN3 stimulated T cells. LN3 cells were stimulated with the corresponding peptides and then further expanded in IL-2 for 1 week. Equal numbers (50,000) of cells from each culture were stimulated with OKT3 + TPA for 16 h (as described in the attached paper, (Fisk et. al.) and the supernatant collected and the cytokine concentration determined by ELISA.

Figure 4. HER-2 peptide induced proliferation and determinant spreading from PBMC of healthy Donor A (A, B). Proliferative responses at primary stiumulation on day 5(A) and Day 6(B). (C, D). Proliferative responses at the secondary stiumlation of primary stimulated cells for 5 days (C) or 6 days (D). The symbols on the upper side of each box indicate the peptide used for primary stimulation. The symbols below each box indicate the peptide used for secondary stimulation.

Figure 5. IFN-γ and IL-4 secretion by secondary stimulated PBMC from Donor A. Autologous plastic adherent APC were used. Proliferation levels shown in **Figure 4C**. Supernatants were collected on day 4. Note the high IFN-γ levels in response to F13 → F7.

Figure 6A, B. Proliferative and cytokine responses of PBMC from Donor B. ($DR1101^{-}$) showing both lack of responsiveness and determinant spreading. Experimental conditions and symbols designation as shown in **Figure 4**.

Table 1 HER-2 peptides used in this study

The Tyr and Trp italicized in position 3 or 4 may constitute P1 anchors. Similarly, the Val, Leu, and Met italicized in positions 4 and 5 may also constitute P1 anchors.

Peptide code	Position	Sequence															
HA	307–319		P	K	*Y*	*V*	K	Q	N	T	L	K	L	A	T		
F12	449–464	G	I	S	*W*	*L*	G	L	R	S	R	E	L	G	S	G	L
G88	450–462		I	S	*W*	*L*	G	L	R	S	R	E	L	G	S		
F14	474–487	T	V	P	*W*	D	Q	L	F	R	N	P	H	Q	A		
F7	776–788	G	S	P	*Y*	*V*	S	R	L	L	G	I	C	L			
G89	777–789		S	P	*Y*	*V*	S	R	L	L	G	I	C	L	T		
F13	884–899	V	P	I	K	*W*	*M*	A	L	E	S	I	L	R	R	R	F
G90	886–898			I	K	*W*	*M*	A	L	E	S	I	L	R	R	R	

Table 2 Summary of proliferative responses of breast cancer patients to HER-2 peptides

Significant proliferative responses according to Student's *t* test are designated +. Responses not significantly different from those in control are designated −. All patients tested showed significant proliferation to PHA (data not shown). The allelism of the HLA-DQ has been determined and is listed. Values for control cultures that were not stimulated with peptides (NP) are listed as C.

Patient	DRB	DRB	DQB	DQB	NP	HA	G90	G88	PHA	F7	F13	G89	F14
1	3	11	301	201	C	−	+	−	+	+	+	+	+
2	4	7	301	201	C	−	−	−	+	+	−	+	−
3	2	4	303	602	C	−	−	−	+	−	−	−	−
4	2	7	201	502	C	−	+	−	+	+	+	+	+
5	6	7	303	303	C	+	+	+	+	+	−	−	+
6	1	7	303	501	C	−	−	−	+	−	−	−	−
7	2	4	302	602	C	+	±	−	+	+	+	+	+
8	3	4	201	201	C	−	−	+	+	−	−	+	−
9	2	4	302	602	C	−	−	−	+	+	−	+	+
10	2	2	602	602	C	−	−	−	+	−	−	−	−
11	3	4	301	501	C	+	−	−	+	+	−	+	−
12	2	4	301	602	C	−	−	−	+	−	−	+	−
13	3	4	302	604	C	+	+	−	+	−	−	+	−
14	3	6	402	501	C	−	−	−	+	−	−	−	−
15	4	7	301	301	C	−	−	−	+	−	−	−	−
16	6	11	603	604	C	−	+	−	+	−	−	−	−
17	6	6	N.D.	N.D.	C	−	−	−	+	−	−	−	−
18	8	8	301	501	C	−	−	−	+	−	−	+	−
Total						4	6	2	18	8	3	10	4
% positive						22.2	33.3	11.1	100	44.4	16.7	55.6	22.2

Figure 1

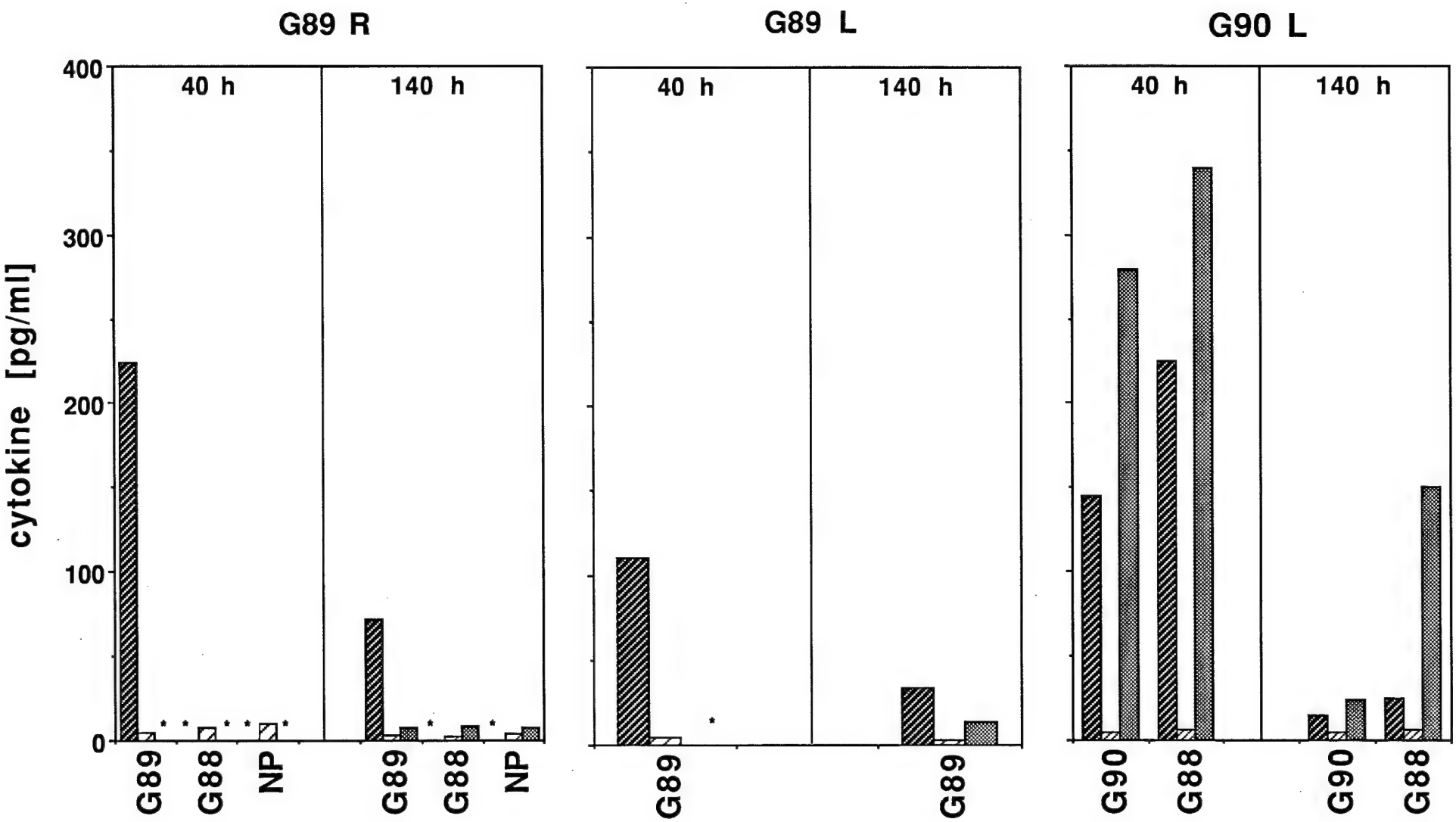

G89 R
G89 L
G90 L
40 h
140 h
cytokine [pg/ml]
400
300
200
100
0
G89
G88
NP
G90

Figure 2

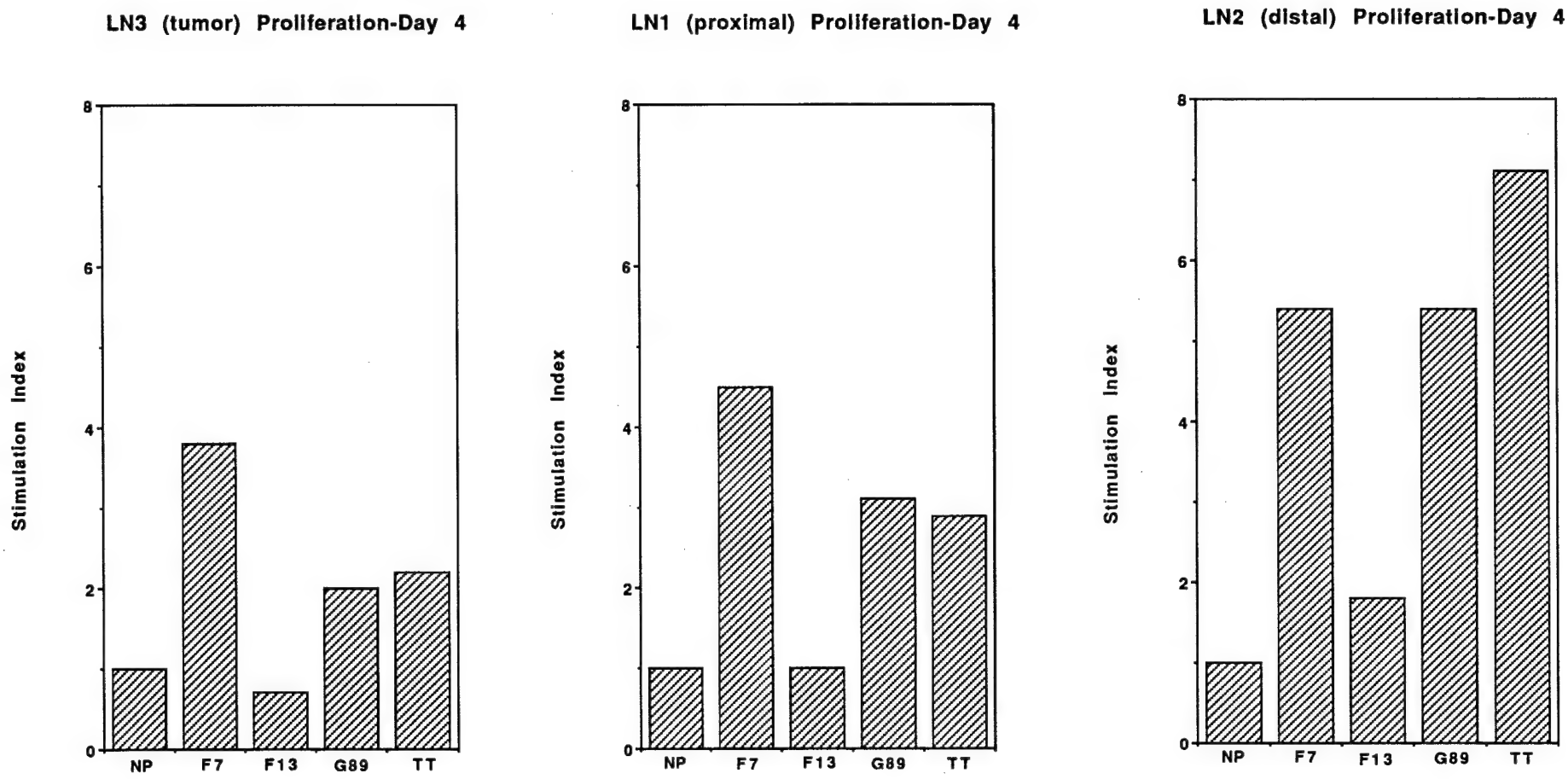
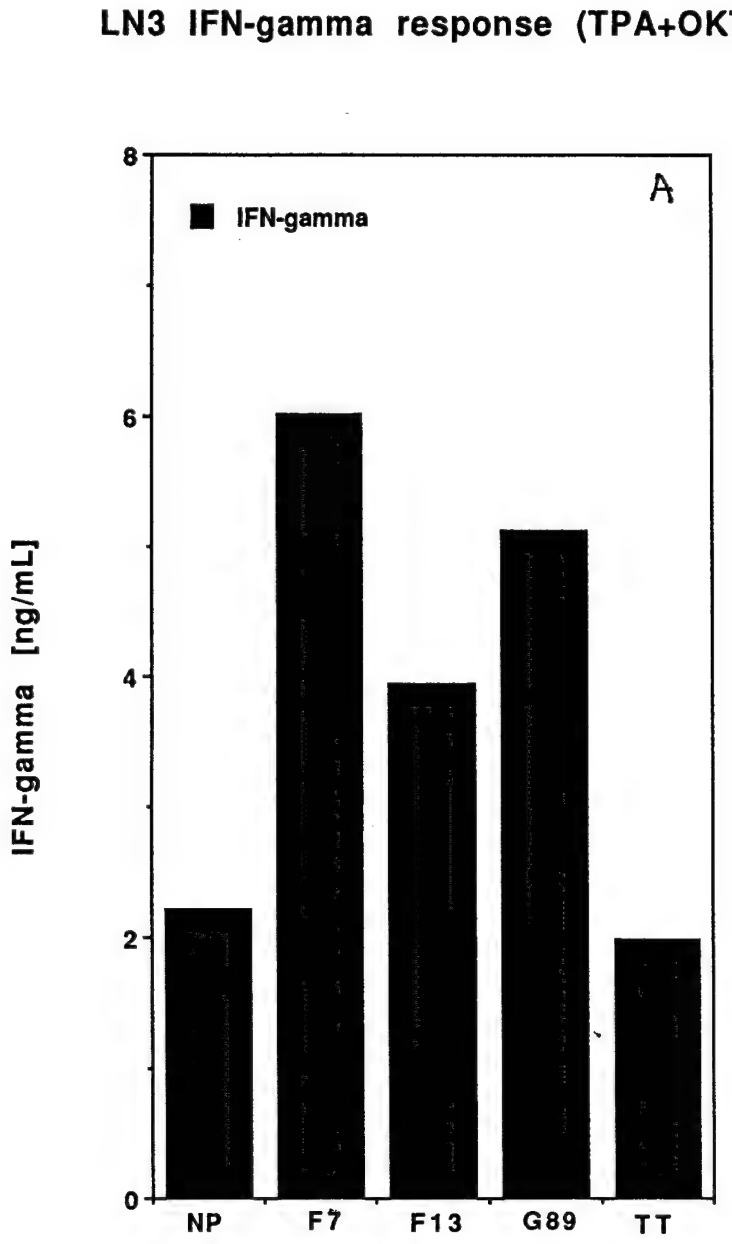
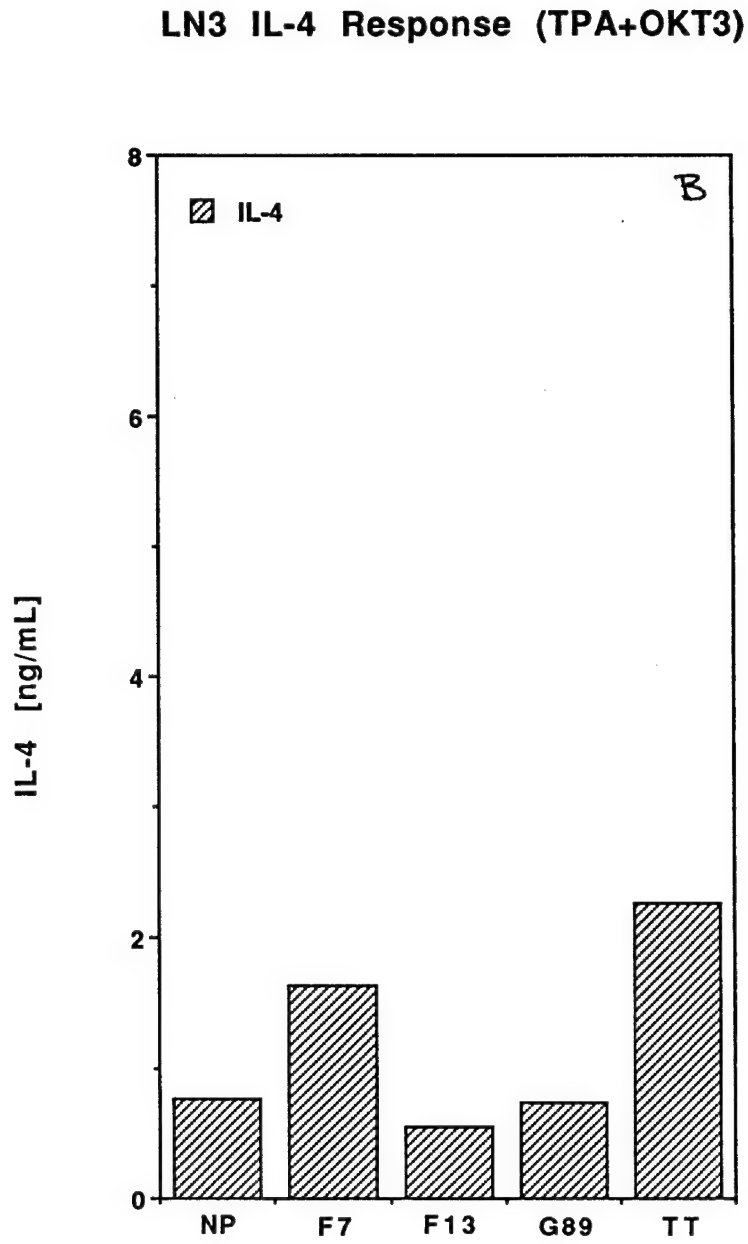

LN3 (tumor) Proliferation-Day 4
LN1 (proximal) Proliferation-Day 4
LN2 (distal) Proliferation-Day 4
Stimulation Index
0
2
4
6
8
NP
F7
F13
G89
TT

Figure 3



IFN-gamma



IL-4

Figure 4

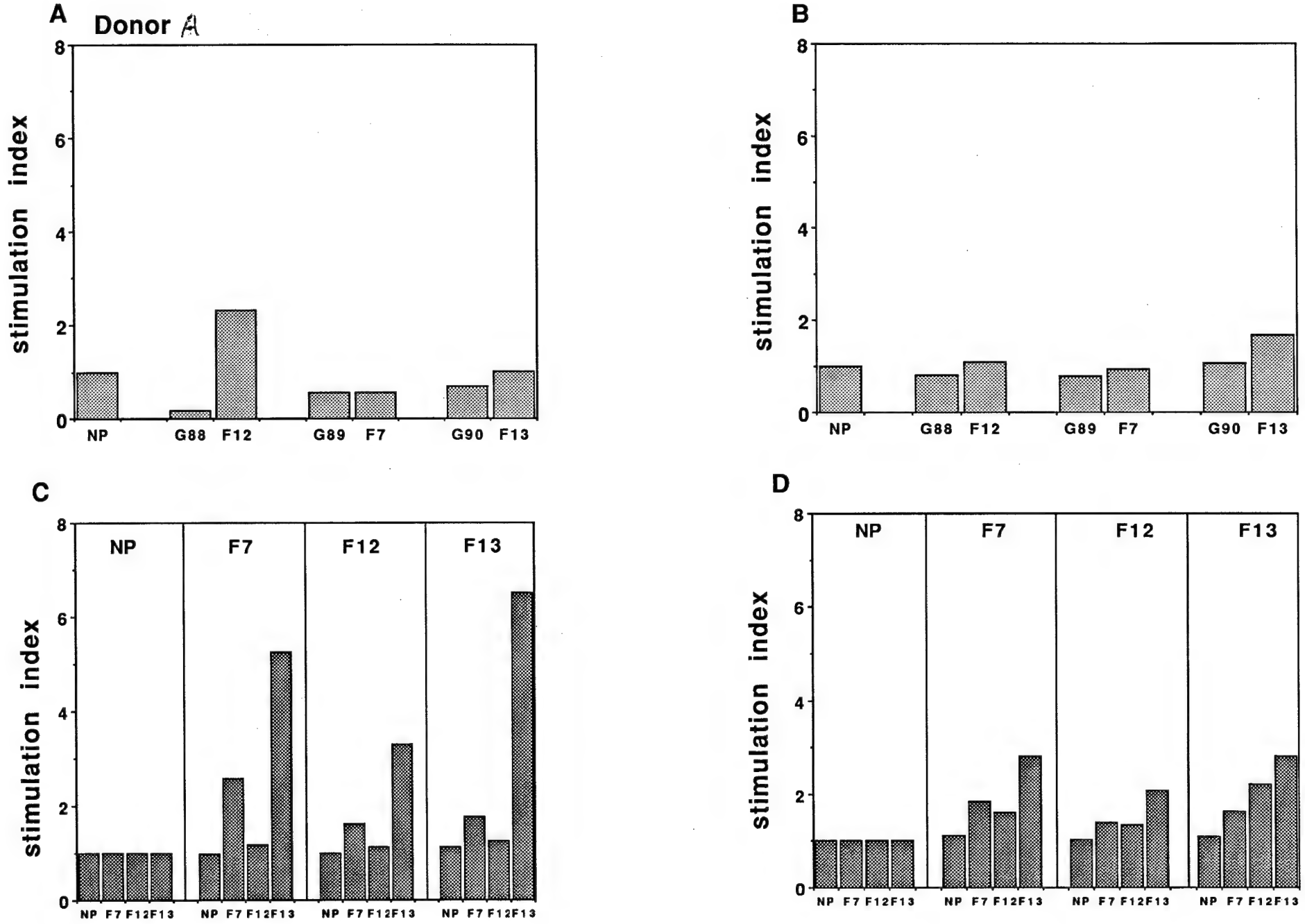

A
Donor A
stimulation index
0
2
4
6
8
NP
G88
F12
G89
F7
G90
F13
B
stimulation index
0
2
4
6
8
NP
G88
F12
G89
F7
G90
F13
C
stimulation index
0
2
4
6
8
NP
F7
F12
F13
NP F7 F12F13
NP F7 F12F13
NP F7 F12F13
NP F7 F12F13
D
stimulation index
0
2
4
6
8
NP
F7
F12
F13
NP F7 F12F13
NP F7 F12F13
NP F7 F12F13
NP F7 F12F13

Figure 5

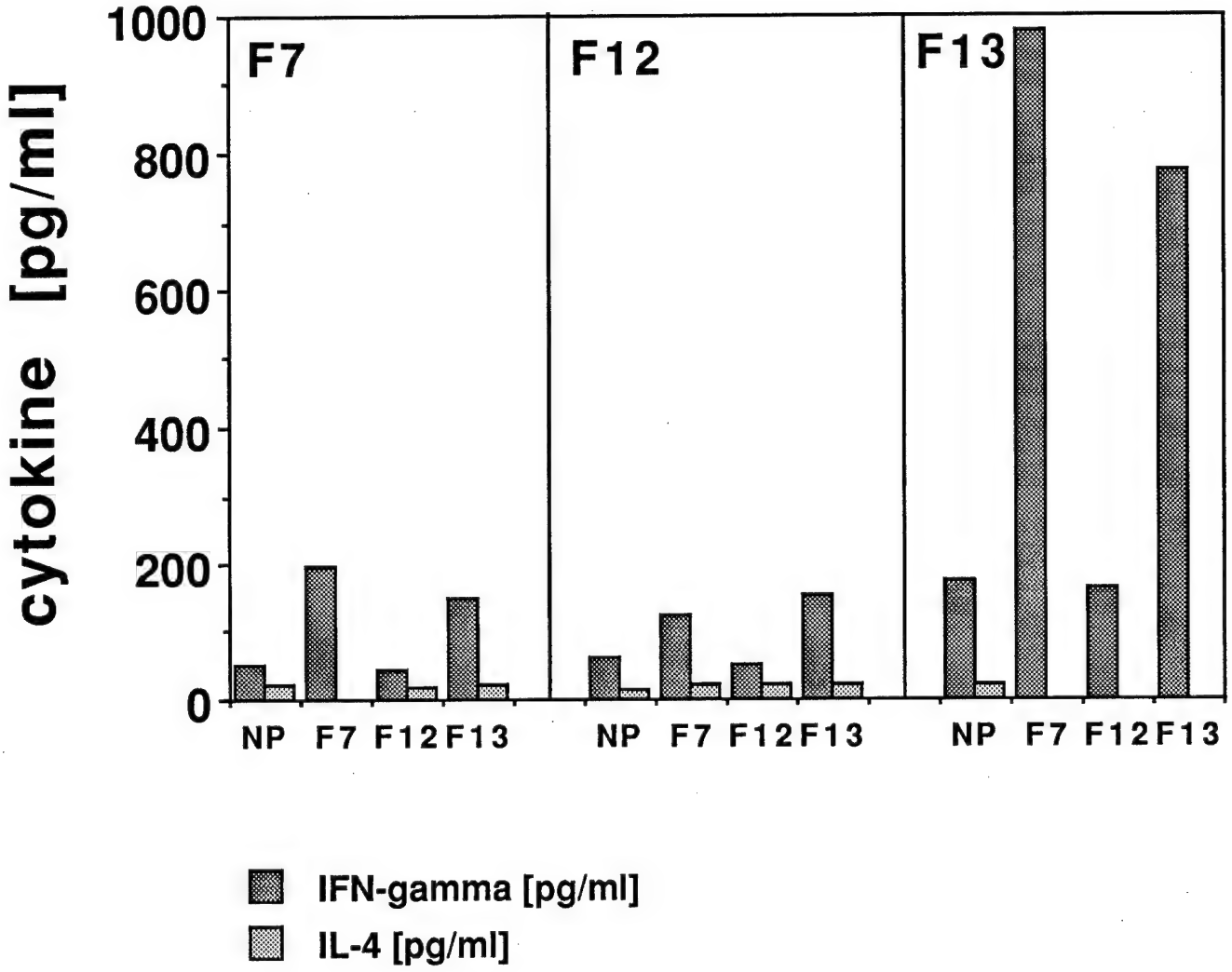
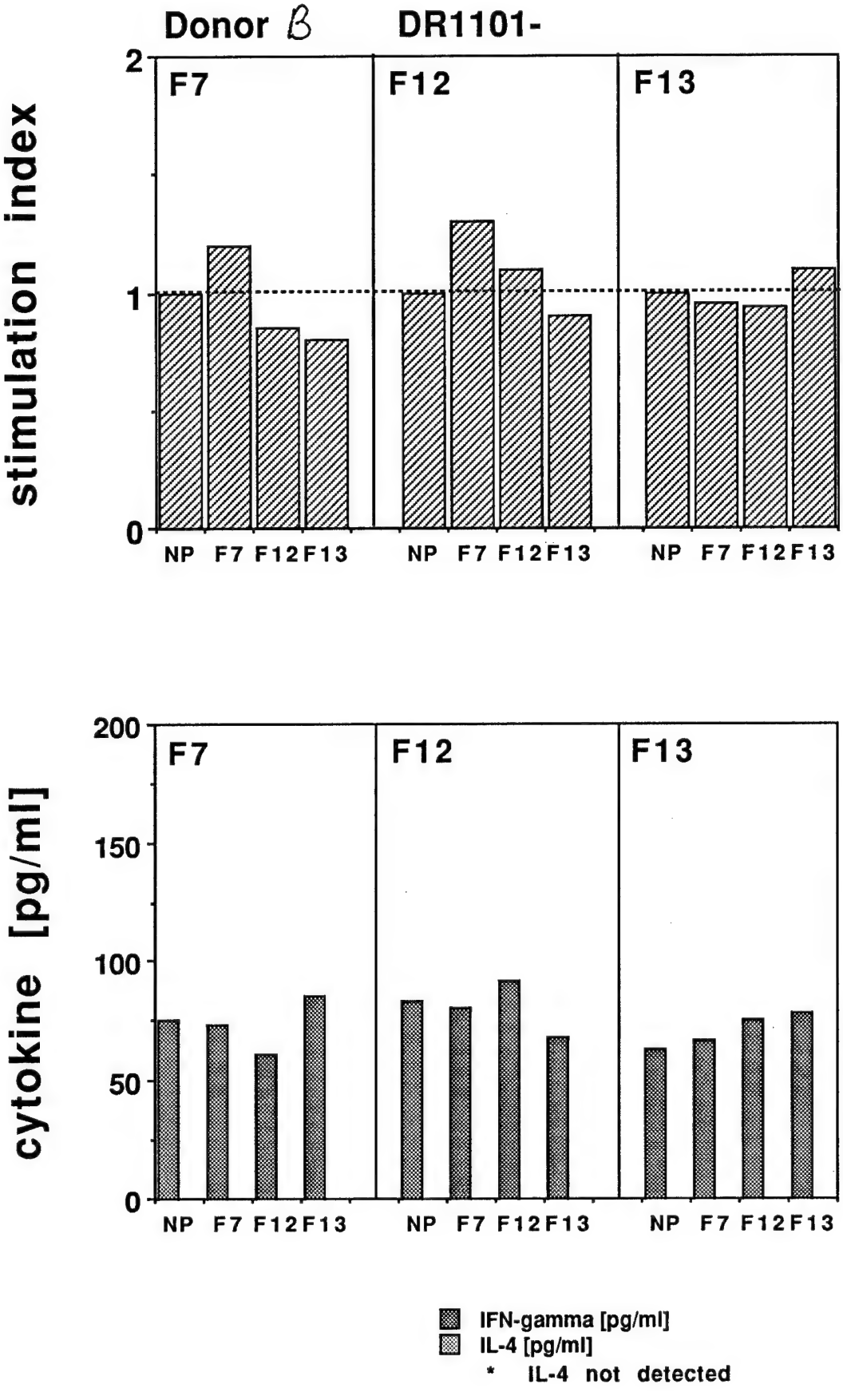

cytokine [pg/ml]
1000
800
600
400
200
0
F7
F12
F13
NP F7 F12 F13
NP F7 F12 F13
NP F7 F12 F13
IFN-gamma [pg/ml]
IL-4 [pg/ml]

Figure 6


Donor B
DR1101-
stimulation index
2
1
0
F7
F12
F13
NP F7 F12 F13
NP F7 F12 F13
NP F7 F12 F13
cytokine [pg/ml]
200
150
100
50
0
F7
F12
F13
NP F7 F12 F13
NP F7 F12 F13
NP F7 F12 F13
IFN-gamma [pg/ml]
IL-4 [pg/ml]
* IL-4 not detected

Publications Resulting from this Grant

1. Fisk, Bryan, Hudson, J. Michael, Kavanagh, John, Wharton, J. Taylor, Murray, James Lee, Ioannides, Constantin G. and Kudelka, Andrzej P. *1997.* Existent Proliferative Responses of Peripheral Blood Mononuclear Cells from Healthy Donors and Ovarian Cancer Patients to HER-2 Peptides. *Anticancer Research 17:45-54.*

2. Tuttle, Todd M., Anderson, Brett W., Thompson, William E., Lee, Jeffrey E., Sahin, Aysegul, Smith, Terry L., Grabstein, Kenneth H., Wharton, J. Taylor, Ioannides[2], Constantin G., and Murray[2], James L., *1998.* Proliferative and Cytokine Responses to Class II HER-2/neu-associated Peptides in Breast Cancer Patients[1]. *Clinical Cancer Research 4:2015-202.*

3. Anderson, B.W., Pollack, M.S., Kudelka, A.P. and loan C.G. M.D. Anderson Cancer Center, Houston, TX 77030, 1998. Induction of Determinant Speading in DR1101+ T cells Peptides Corresponding to HER-2 Helper Epitopes Results in a Th1 Cytokine Response. *Proceedings of the American Association for Cancer Research 39:84-85, A574.*

ANTICANCER RESEARCH *17*: 45-54 (1997)

Existent Proliferative Responses of Peripheral Blood Mononuclear Cells from Healthy Donors and Ovarian Cancer Patients to HER-2 Peptides*

BRYAN FISK[1], J. MICHAEL HUDSON[1], JOHN KAVANAGH[2], J. TAYLOR WHARTON[2], JAMES LEE MURRAY[3], CONSTANTIN G. IOANNIDES[1] and ANDRZEJ P. KUDELKA[2]

The Departments of [1]Gynecologic Oncology, [2]Clinical Investigations, Section of Gynecologic Medical Oncology, and 3Bioimmunotherapy, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, Texas 77030, U.S.A.

Abstract. *Identifying target antigens for tumor-reactive T cells is important for understanding the mechanisms of tumor escape and developing novel anticancer therapies. To date, mainly CTL responses from tumor infiltrating/ associated lymphocytes (TIL/TAL) to peptide antigens have been investigated in ovarian cancer. In the present study, the ability of self-peptides derived from HER-2/neu proto-oncogene product (HER-2) to stimulate proliferation of PBMC from healthy donors and ovarian cancer patients has been assessed. Peptide sequences from HER-2 containing anchors for major human MHC-class II molecules have been identified. These peptides induced proliferative and cytokine responses at higher frequency in healthy donors than ovarian cancer patients. Four HER-2 peptides corresponding to positions: 396 - 406, 474 - 487, 777 - 789, and 884 - 899 were able to stimulate proliferation of a larger number of healthy donors than three other distinct HER-2 peptides 449 - 464, 975 - 987 and 1086 - 1098. The pattern of responses of twenty five ovarian cancer patients was different from that in healthy donors. T cell lines were developed by stimulation with peptides from PBMC of an ovarian cancer patient who showed a stable response to all four HER-2 peptides for over six months. Each T cell line was different in its ability to secrete IFN-γ and IL-10. These results demonstrate (a) that self-peptides from HER-2 can stimulate expansion of T cells in both healthy donors and ovarian cancer patients, and (b) the ability of different peptides to stimulate secretion of different cytokines from lymphocytes of ovarian cancer patients. These results may be important for understanding the mechanisms of tolerance and autoimmunity in human cancers.*

The HER-2/neu proto-oncogene product (HER-2) is the target of autoantibodies in breast cancer (1) and of cytotoxic T lymphocytes (CTL) in ovarian, breast, and lung cancer (2-5). Since these auto-antibodies are specific for the native conformation of the HER-2, they must be induced by the native molecule. HER-2 is present in both healthy individuals and cancer patients. Similarly HER-2-reactive CTL are specific for a number of epitopes (2-5) of which one HER-2, 369-377 was found to be immunodominant in our studies (5). These CTL were isolated from lymphocytes associated with ovarian tumors in patients with advanced disease after culture in the presence of IL-2. This suggests that $CD4^+$ T cells capable of helping either B cells, or $CD8^+$ CTL, or both may be present in the cancer patients. This raises the question as to whether CD4+ T cells capable of recognizing epitopes from human HER-2 are also present. Previous studies have

Abbreviations: Cytotoxic T Lymphocytes, CTL, Position, P; T cell receptor, TCR; HER-2/neu proto-oncogene, HER-2; stimulation index, S.I.; standard deviation, SD, TT, tetanous toxoid.

**Acknowledgments.* We thank Ms. Rosario Mante for collecting specimens and Ms. Susan Mondragon for the outstanding preparation of this manuscript. Peptide synthesis was supported in part by core grant 16672.

Correspondence to: Dr. Constantin G. Ioannides, Department of Gynecologic Oncology, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Box 67, Houston, Texas 77030, USA.

Key Words: HER-2, $CD4^+$, epitope, ovarian cancer, Th1, Th2, cytokines.

Table I. *Sequences of HER-2 peptides.*

PeptideCode	Position	Sequence[a]
F11	5- 19	A L C R W G L L L A L L P P G
D122	396 - 406	Q L Q V F E T L E E T
F12	449 - 464	G I S *W* L G **L** R S **R** E **L** G S G L
F14	474 - 487	T V P *W* D Q *L* F *R* N P H Q A
F7	776 - 789	G S P *Y* V S **R** L **L** G I C L
F6	777 - 797	G S P *Y* V S **R** L *L* G I C L T S T V Q
F8	832 - 851	G M S *Y* L E D V R L V H R D L A A R N
F10	975 - 997	F S R *M* A R S P Q R *F* V V **I** Q N E D **L** G
D100	1086-1098	F D G D L M G A A K G L
F13	884 - 899	V P I K *W* **M** A L **E** S **I** L R R R F

[a]Potential DR4 anchors that distinguish between DRB1*0401/0404 and DRB 1*0402 are shown in bold. Tyr (Y) and Trp (W) residues characteristic of the P1 anchors for DRB1*0401 and DRB*0101 binding motifs (21) are italicized. Prolines for protection from proteolysis are underlined. Peptide F10 extends a potential helper epitope after the CTL epitope C85 (the sequence is underlined). Both DR4 allotypes (DR4.1 and DR4.2) accept peptides with Leu, Ile, Met, Phe, and Val as P1 anchor residues (19).

shown that $CD4^+$ T cells with specificity for HER-2 can be identified in breast cancer patients (1). The extent and the existence of autoreactive T cell repertoire to HER-2 in both healthy humans and ovarian cancer patients has not been previously identified. To assess the specifIcity of these T cells and identify potential targets for epitope-specific immunotherapy, we investigated the responses to HER-2 of a group of patients with ovarian cancer subsequent to chemotherapy and a group of healthy individuals.

To characterize the T cell response to HER-2 we assessed a number of T cell epitopes of HER-2 with a set of synthetic peptides based on the HER-2 sequence. We wanted to identify a set of such peptides to which healthy donors and ovarian cancer patients respond by proliferation and determine the frequency of these responses. We stimulated PBMC from twenty five ovarian cancer patients and fourteen healthy donors with synthetic HER-2 peptides. PBMC from each donor were stimulated individually with each peptide, but not with pooled peptides. The general pattem of response was characterized by a group of four HER-2 peptides designated as D122:HER-2,396 - 406, F7:HER-2:777 - 789 F13:HER-2,884 - 899, F14:HER-2, 474 - 487 which induced a signifIcantly higher frequency of responses than the other three HER-2 peptides designated as F10:HER-2,975 - 997, F12:449 - 464, D100:HER-2,1086 - 1098 in both healthy donors and ovarian cancer patients. The frequency of responses to most HER-2 peptides was significantly lower in ovarian cancer patients who had received chemotherapy than in healthy donors.

T cell lines were raised against individual HER-2 epitopes represented by peptides F7, F13, F14 and D122 from PBMC of an ovarian cancer patient by restimulation with HER-2 peptides and expansion in IL2. These T cell lines showed a different pattern of IFN-γ and IL-10 production. F13 induced T cells secreted significantly higher amounts of IFN-γ than IL-10 while F7 and F 14 induced T cells secreted significantly higher levels of IL-10 than F13 and D122 induced cells.

Materials and Methods

Cells. Peripheral blood nononuclear cells (PBMC) were obtained from fourteen healthy donors and twenty five ovarian cancer patients. All patients had advanced disease. After initial surgery, they were treated with platinum (cisplatin or carboplatin). One patient was receiving primary platinum based chemotherapy. The other twenty four patients had received additional chemotherapy. The latter was either carboplatin reinduction, salvage therapy with paclitaxel or experimental therapy with several different drugs. Blood collection was made at least three weeks after the last chemotherapy administration. PBMC were isolated from heparinized peripheral blood as described (1,6). At the time of the assay the patients were not receiving chemotherapy.

HER-2 epitopes selection. Peptides to be tested in the proliferation assays were selected based on the T cell sites in HER-2 predicted by the

computer program ANT.FIND.M, the general binding motif for human class MHC-II antigens, and the presence in the sequence of anchors for a number of MHC-class II antigens: HLA-DRl, HLA-DR3, HLA-DR4, HLA-DRll, and HLA-DQ7 (7-13). The sum of the allelic frequencies of these antigens cover approximately 100% of the Caucasian and Hispanic populations and between 75 - 92% of the African American and Asian populations. For example HLAD97 is present at 28% (Caucasians) 23% (African Americans), and at 43% (Hispanics). Similarly, each of HLA-DR1, DR3, DR4 and DR11 is present between 17 - 20% in each of the major population groups. The general binding motif for various human MHC-class II molecules consist of a position 1 (P1) anchor, *i.e.* an aromatic or large aliphatic residue in the first 3 - 5 amino acids close to the N-terminus, and other major but less essential anchors at positions 4, 5 - 7, and 9 counting from the Pl anchor (13). A large number of "promiscuous" peptides are capable of binding to many different MHC-class II molecules (13, 14), because their sequences contain overlapping binding motifs for MHC-class II molecules (13, 14). The search for specific anchors for these major MHC-class II antigens in the HER-2 sequence indicated significantly more candidate epitopes for binding to HLA-DR1 (n = >20) than for HLA-DR3 (n = 11), than for binding to HLA-DR4, HLA-DQ7, and HLA-DR11.

Ten HER-2 peptides 11 - 22 residues long (Table I) were synthesized by the Synthetic Antigen Laboratory of M.D. Anderson Cancer Center, using a solid phase method as previously described (2). The identity of peptides was determined by amino acid analysis. The purity of peptides ranged between 93 - 97% as determined by HPLC. Peptides were dissolved in PBS, aliquoted at 2 mg/ml and stored frozen at -20°C until use. The codes used to identify these HER-2 peptides in this paper were assigned by the Synthetic Ag Laboratory. To ensure better representation of different binding motifs for these MHC class II antigens, at least two peptides were synthesized containing anchors for each of HLA-DR1, -DR3, and -DQ7. When possible the sequences were selected to contain anchors for two MHC class II antigens (Table I). Each of the peptides synthesized contained at least two of three anchors for each HLA-DR antigen, as shown in the Table I, and the main Pl anchors for most class II alleles. In peptides D122, F12, F7, F6, F8 and F13, positions P4 and P5 are occupied by hydrophobic, aromatic followed by aliphatic residues in that order to facilitate peptide binding in different frames.

The binding motifs of synthetic peptides for MHC-class II molecules may differ from natural ligands because the latter incorporate constraints of processing in addition to binding requirements. For these reasons, peptides were synthesized by following, when possible, the common motifs for all MHC-class II molecules defined by pool sequencing of naturally processed peptides (7). This was possible for four peptides, F6, F7, F13 and F14. In these peptides, the sequence was extended to include Pro (P) N-terminal to either the Tyr (Y), which is the P1 anchor for HLA-DR1, DR3, DR4, and DQ7, or Trp (W) which is reported by the Pl anchor for HLA-DR4 and DR1 1. Peptides F6 and F7 overlap in the first thirteen residues. In F6 the sequence was extended at its C-terminal to incorporate a region 783-797 previously reported to induce proliferation of PBMC from breast cancer patients. The sequence of F14 was also extended to include Pro at the C terminus, after Arg (R), the third anchor in the correct position for HLA-DR3 and HLA-DR1 1. Sequences were also extended at the N- and C-termini. This was made to facilitate the natural proteolytic trimming of peptides since most aminopeptidases stop cutting one residue before reaching a Pro residue (7).

Stimulation and propagation of T cells. Freshly separated PBMC from healthy donors and ovarian cancer patients were stimulated with each HER-2 peptide at a final concentration of 50 μg/ml and cultured at 1×10^6 cells/ml in RPMI 1640 medium (GIBCO) with 5% pooled human AB serum and antibiotics (complete RPMI medium) (1). After 3 - 4 days of stimulation with each peptide, cultures were expanded with IΛ-2 (20 - 40 U/ml) for the following week (15, 16). To induce antigen specific T cells, the cells were then "rested" for 3 - 4 days by culture in

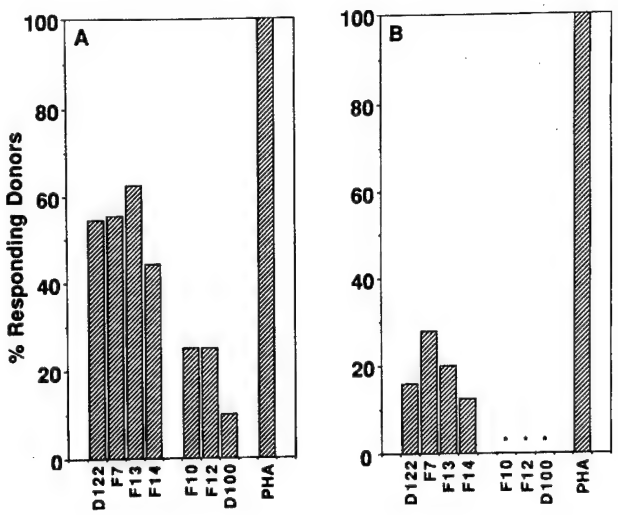


Figure 1. *Histograms of positive blastogenic responses of PBMC from (A) 14 healthy donors and (B) 25 ovarian cancer patients to seven HER-2 peptides of the sequences listed in Table I. The Y axis indicates the fraction of donors with a positive response to each peptide. A blastogenic response was considered significant when the p values for peptide stimulated vs. control cultures were <0.05. Stimulation indexes (S.I.) were obtained by dividing the means of cpm proliferation of PBMC in the presence of peptides with the means of cpm proliferation in the absence of peptide. S. I. for the peptides inducing significant proliferation ranged between 1.5- 3.0, while for the peptides that did not induce significant proliferation ranged between 0.9 - 1.2. Proliferation values (cpm + SD) are shown in Figures 2 and 3. Peptides F7 and F13 elicited the most positive responses in PBMC from both healthy donors and patients (at least 5 donors positive in each group). In (B), (*) the frequency of responses to F10, F12, and D100 respectively were as follows: F10: 1/25, F12, 1/25 and D100: 2/25.*

the absence of IL-2. Then, the cells were stimulated at a 1:1 (stimulator:responder ratio) with irradiated (10.000 Rad) autologous PBMC, which had been flrst stimulated with PHA, expanded in IL-2, and then were pulsed with individual peptides for at least 90 min at 37°C before addition to the cultures as described (16). Control cultures were stimulated with the same number of IL-2 expanded PBMC in the absence of peptides. For further expansion, four to five days later 20 - 40 U/ml IL-2 was added to the cultures for seven additional days.

Proliferation assays. The proliferation assay was done by culturing 2×10^5 PBMC from each donor in quadruplicate in a 96 well plate in 200 μl with each peptide at 50 μg/ml, with tetanus toxoid at 5 μg/ml and PHA (GIBCO) at a final concentration 1:100 for 96 h as described (1, 16). For the last 16 h, 1 μCi [^{3}H]-Tdr was added to the cultures. Afterwards, the cells were harvested and the radioactivity counted in a Beckman LS3501 liquid scintillation counter as previously described (6). A significant proliferative response was deflned as a statistically significant increase in the cpm proliferation in the tetraplicate cultures stimulated with any of the peptides, PHA, or TT, above that in cultures from the same donor that received peptide. Values obtained for cpm (^{3}H-Tdr incorporation) by the PBMC incubated with PBS, PHA or synthetic HER-2 peptides were examined by the Student's t test. Differences were considered significant when the P values were < 0.05.

Flow cytometry. T cells were tested in fluorescence experiments to determine the surface antigen expression as previously described (2, 6).

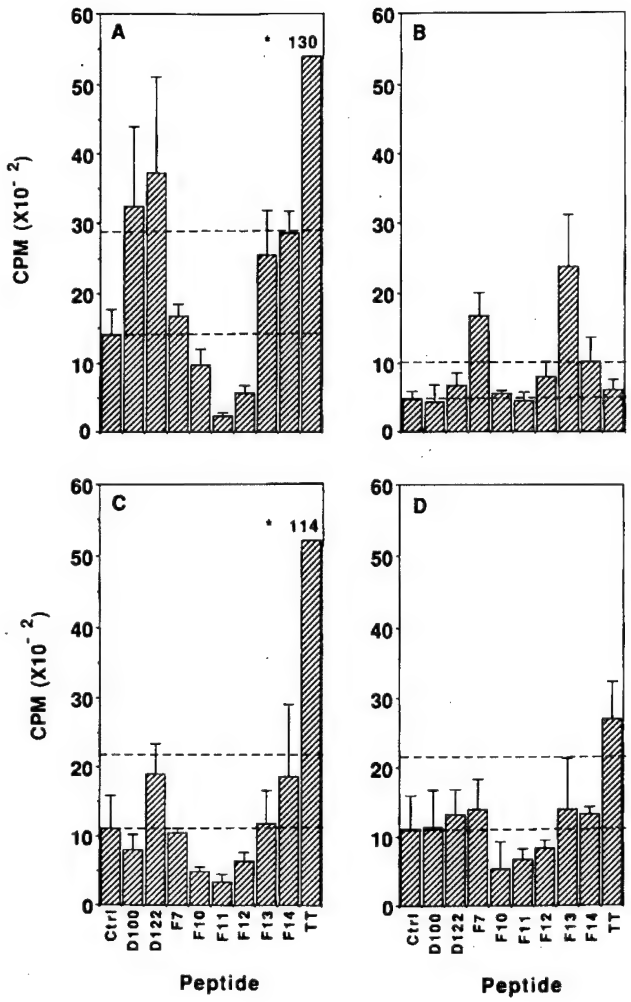


Figure 2. *Proliferative responses to HER-2 peptides by PBMC from four healthy donors A, B,C,D determined in the same experiment. Two responded to peptides as follows: Donor A to D 100, D 122, F13 and F14; Donor B to F7 and F13. Two were non-responders (panels C, D). Results indicate cpm ± SD. Dotted lines indicate proliferation corresponding to S.I. of 1.0 and 2.0 respectively. *cpm proliferation to TT not on scale.*

Surface antigen expression was determined by FACS analysis using a FACSscan (Beckton-Dickinson, Sunnyvale, CA)) with a log amplifier. CD3, CD4 and CD8 antigen expression on T cell cultures was determined by immunofluorescence with corresponding mAb FITC-conjugated (Beckton Dickinson).

Cytokine production. The ability of PBMC to produce antigen induced IFN-γ and IL- 10 was determined by culturing the PBMC either as unstimulated or stimulated with the corresponding peptides or PHA, (GIBCO) diluted 1:100, or tetanus toxoid. Supematants were collected after 48h and stored frozen at -20°C until assayed for cytokine level. Cytokine containing supematants from the T cell lines were generated by adding OKT3 and phorbol myristate acetate (PMA) to the cells to 96 well plate for 18h as described (17, 18). Afterwards, supernatants were collected for measurement of IFN-γ and IL-10 levels. IFN-γ and IL- 10 were measured by double sandwitch-ELISA using the corresponding kits provided by BioSource International (Camariyo, CA). Supematants from tetraplicate wells were pooled and tested in triplicates. The cytokine assays were calibrated with human recombinant IFN-γ and IL-10 to detect each cytokine in the range of 10-1000 pg/ml.

Results

Proliferative responses of PBMC to HER-2 peptides. To map the HER-2 peptides most frequently recognized and to identify potentially immunodominant epitopes, we determined the responses to individual peptides of PBMC from twenty five ovarian cancer patients. Patients previously treated with chemotherapy were allowed on this study, since immunotherapy approaches to ovarian cancer are usually initiated after conventional therapies thus making the responses of these donors more likely to reflect the responder status of candidates for tumor-vaccine therapies. Responses of PBMC from healthy donors to the same peptides were tested in parallel in the same experiment. Depending on the number of PBMC available from each ovarian cancer patient a minimum of four peptides and PHA were tested in the same experiment. Results are summarized in Figure 1. Peptides Fl 1 (HER-2, 5-19) (which corresponds to the highly hydrophobic signal area) F6 (HER-2, 777-797) and F8 (HER-2, 832 - 851) were not easily solubilized in PBS, thus their use for stimulation was discontinued after several assays. PBMC from most donors were tested with at least six peptides in each experiment. Each donor responded to some peptides but not to others. The lack of a common response it is not entirely suggesting that these peptides do not bind MHC molecules. A negative response of any of these peptides could also reflect T cell unresponsiveness (tolerance) to this epitope.

To ensure that the lack of responsiveness of PBMC to HER-2 peptides did not reflect a generalized suppression of responses to antigen or mitogen, all patients' lymphocytes were tested for their ability to respond to PHA. Of 29 patients tested, 28 showed significant responses to PHA compared with unstimulated cultures. All PBMC samples from healthy donors showed significant responses to PHA (data not shown). Together these results indicated that most PBMC from the ovarian cancer patients with advanced disease respond to PHA after chemotherapy and that their ability or failure to respond to HER-2 peptides did not reflect their ability to respond to a T-cell mitogen.

The frequency of responses of healthy donor PBMC to four peptides: D 122, F7, F13 and F14 was higher than the frequency of responses to three other peptides, F10, F12, and D100. The same pattem of preferential responses to F7 and F13 was seen in PBMC from ovarian cancer patients (Figure lB). The results also indicate that PBMC from ovarian cancer patients responded at a significantly lower frequency than healthy donors to F7, F13, F14 and D122. However, the decrease was not the same for all peptides. For peptides D122, F13, and F14 the decrease in responses in PBMC from patients versus healthy donors was in the range of 70%, while for peptide F7 the decrease was only 50%. No significant

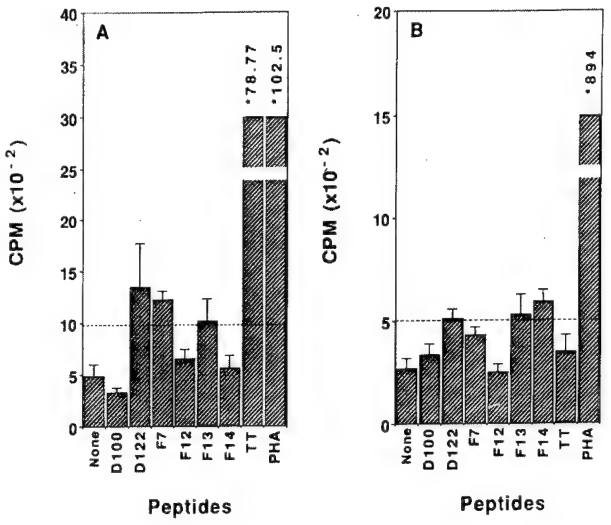


Figure 3. *Proliferative responses to HER-2 peptides of PBMC determined in the same experiment. (A) ovarian cancer patient; (B) healthy donor. Significant differences between peptide stimulated and no peptide cultures were observed in (A) for D122, F7 and F13 and in (B) for D122, F13 and F14. Dotted lines indicate proliferation corresponding to S.I. of 2.0.*

responses to peptides D100 (1086 - 1098), F10 (975 - 987), and F12 (449 465) were observed. Representative results from PBMC of four healthy donors (two responders and two non-responders) are shown in Figure 2. Donors A and B were considered responders based on the ability of their PBMC to proliferate to at least one of the eight HER-2 peptides tested. These responses were significantly higher than responses by PBMC that have not been pulsed with peptides. In both responders and nonresponders, the responses to HER-2 peptides did not correlate with the ability of the same PBMC to respond or with the magnitude of response to TT (Flgures 2 and 3). Of twenty flve ovarian cancer patients tested, seven responded to F7 with statistically signiflcant differences in cpm proliferation between peptide-induced and control cultures. Of seven F7 responding patients three responded to F13 two responded to F7, F13 and F14; one to F7 and F14; and four only to F7. Of five F13 responding patients two did not respond to F7 or Fl4. Only two patients responded to all four peptides: F7, F13, F14 and D122. There was no signiflcant difference in the magnitude of their proliferative responses to these four peptides.

For comparison, the pattem and the magnitude of the proliferative responses to HER-2 peptides of PBMC one of these patients (designated as Patient A) is shown in Flgure 3 together with those of PBMC from another healthy donor tested in the same experiment. Both donors responded to D122, F7, F13 and F14 but failed to respond to D100 and F12. To establish whether the pattern of responses to the HER-2 peptides in PBMC from ovarian cancer patients changed over time, responses to the same peptides were determined at two additional time points over five months when the patients were not receiving chemotherapy. PBMC from Patient A showed a constant high level of responses to at least two of the four peptides tested, but PBMC from a nonresponding patient failed to proliferate above the control levels. The same PBMC showed significant responses to PHA. It should be mentioned that sixteen months after the first determination, PBMC from the same Patient A responded primarily to F13 also less strongly to F7 (data not shown). PBMC from the same responding patients were tested again after several months when the disease progressed. At this time significant proliferative responses to HER-2 peptides were not observed. All three patients that responded to both F7 and F13 had stable disease. This suggests that disease progression affects the ability of PBMC from cancer patients to respond to HER-2 peptides.

In vitro expansion and cytokine production by HER-2 peptide stimulated T cells. To establish whether HER-2 peptide stimulated lymphocytes can be expanded in culture as T-cell lines, PBMC from Patient A were selected for these experiments. These PBMC were chosen because they showed a stable and significant proliferative response to at least four HER-2 peptides of distinct sequence. Primary cultures with F7, D122, F13 and F14 were initiated for four days, after which IL-2 (40 U/ml) was added for four more days. Afterwards, IL-2 was removed and the cells were "rested" in complete RPMI medium in the absence of IL-2 for four days. Afterwards, each peptide initiated culture was restimulated with autologous T cells from PHA-stimulated PBMC expanded in IL-2 prepulsed with the corresponding peptide. Control cultures were then restimulated with PHA blasts in the absence of peptides. After four days, IL-2 (20 U/ml) was added to the cultures for 48 h. The S.I. were determined by comparing the 3H-Tdr incorporation during the last 16 h. As shown in Figure 4A all peptide stimulated cultures showed an increase in S.I. of >2.0 over the control cultures. Similarly, cultures stimulated initially with TT, PHA and OKT3 in the presence of IL-2 showed a significant increase in proliferation over the peptidestimulated cultures.

To address whether T cells induced by one of the peptides can be stimulated to proliferate by another HER-2 peptide, we investigated the response of F13-induced T cells to F13, D122, F14, and F7. The results are shown in Figure 4B. PHA and OKT3 mAb were used for stimulation as positive controls. Cells were counted after four days. A signiflcant increase in number over control cultures was observed in F13 induced cultures restimulated with F13. In contrast, F13-induced cultures showed no signiflcant proliferation in response to F14, and F7 while in response to D122 the viable cell number actually decreased. These results indicate that F13-induced T cells preferentially proliferate in response to F13 and are only minimally cross-stimulated to proliferate by other HER-2 peptides.

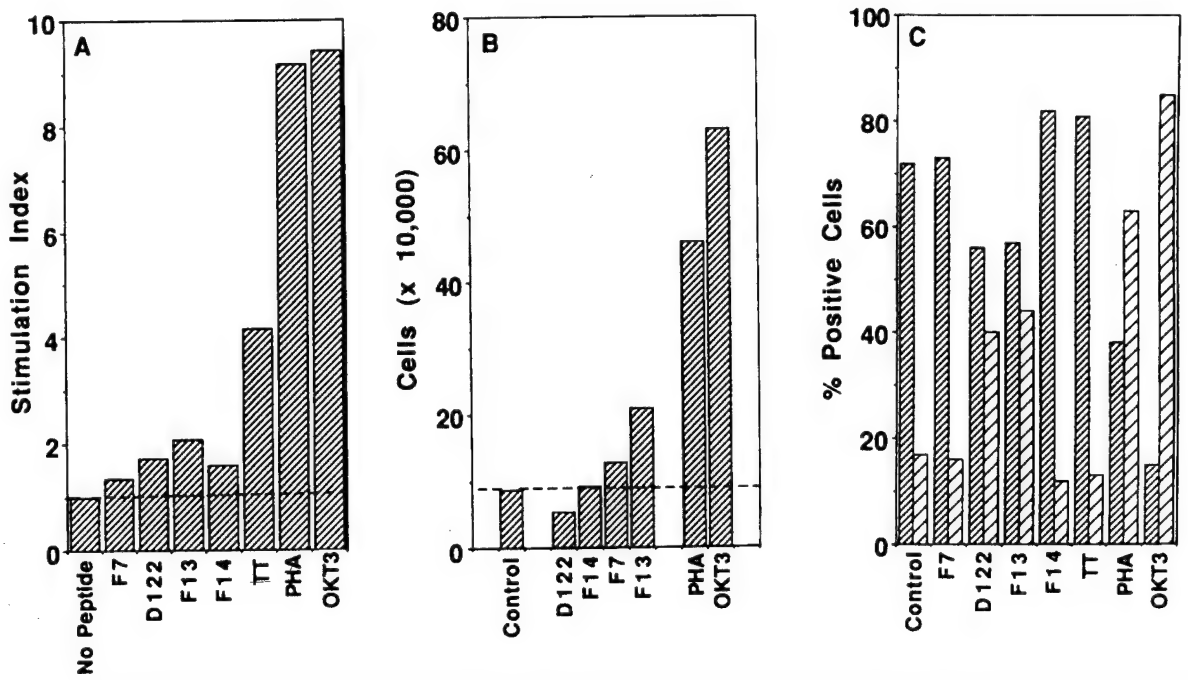


Figure 4. *(A) Proliferative responses to HER-2 peptides F7, F13, F14, and D122 by T-cell cultures induced with the corresponding peptide and expanded in IL-2. (B) Increase in cell number of F13 induced T cells described in (A) after restimulation with either no peptides (control), each of the peptides D122, F14, F7 and F13; or PHA and OKT3 mAb. Experimental conditions were as described in the Materials and Methods. (C) Cell surface phenotypes of peptide-induced cultures described in (A), after restimulation with each peptide and expansion in IL-2 ▨ $CD4^+$ cells, ▨ $CD8^+$ cells.*

To define the T cell phenotype, the resulting PBMC cultures, were analyzed for CD4 and CD8 antigen expression. Analysis of the phenotype of the control cultures was performed in parallel. The results are shown in the Figure 4C. Most cells in the primary stimulated cultures were $CD4^+$ T cells, however there were some significant differences between cultures. F7- and F14-stimulated cultures contained 12 and 16% $CD8^+$ cells respectively, while D122 and F13-stimulated cultures had a significantly higher proportion of $CD8^+$ cells (40 - 44%). Control cultures which were stimulated only with autologous PBMC and IL-2 were of predominantly of the CD4 phenotype while in PHA and OKT3 stimulated cultures $CD8^+$ cells were in majority. These results indicate that, for the same donor, stimulation with each peptide affected in a different way the proliferation of either $CD4^+$ or $CD8^+$ cells or both. This does not reflect the ability of one or another subset to proliferate better in the presence of IL-2. Control cultures which were not stimulated with exogenously added peptides, and TT-stimulated cultures showed a predominantly $CD4^+$ phenotype. Inhibition studies using anti-HLA-mAb indicated that HER-2 peptides induced proliferation was inhibited by anti-MHC class II and at lesser extent by anti-MHC-class I Abs suggesting that the responder cells are T cells (data not shown).

Each set of HER-2 stimulated lymphocytes showed different proportions of $CD4^+$ and $CD8^+$ cells. Since this may reflect the ability of each peptide to induce cytokines, which can affect the proliferation of each T cell subset, the capacity of each HER-2 induced T-cell line to secrete IFN-γ and IL- 10 was determined. These cytokines have been associated with Thl and Th2 types of responses, respectively (19-21). Since cells cultured in IL-2 usually produce background levels of IFN-γ, all peptide-stimulated T-cell lines and control lines were washed and cultured in complete RPMI medium without IL-2 for two days, before being stimulated with OKT3 and PMA. The results of one representative experiment (of two experiments performed) are presented in Figure 5.

T-cell lines stimulated by F7, F13, and F14 produced IFN-γ at higher levels than observed in control cultures stimulated with autologous PBMC but not with peptides. The highest levels were observed with F13 and were similar to the levels induced by TT-induced T cell lines. Interestingly, a different pattern of IL-10 secretion was observed. IL-10 secretion by F13-stimulated T cells was only slightly above the control levels. However, high levels of IL-10 were found in the supernatants from F7- and F14-stimulated T cells. The levels of IL-10 were almost half the level of IL-10 produced by TT-induced T cells. In contrast, while the levels of IFN-γ produced by D122-induced line were higher than those produced by the control cultures, the levels of IL-10 produced by the D122-induced line were minimal. These results show a good correlation between the IL-10 secretion and the $CD4^+/CD8^+$ ratio of T cells from HER-2 peptide induced T

cell lines. F7-, F14- and TTinduced T-cell lines secreted high levels of both IFN-γ and IL-10. Conversely, D122- and F13-stimulated cultures secreted different amounts of IFN-γ but low amounts of IL-10. This may be suggestive of a Thl function for the F13 peptide in this patient and for a Th2 function for the F7 peptide in the same patient.

Discussion

Recognition of HER-2 epitopes by $CD8^+$ cytotoxic T lymphocytes has been extensively documented (2-5). However significantly less information is available about the recognition of HER-2 epitopes by $CD4^+$ cells. Although $CD4^+$ cells may not be always involved in tumor lysis in breast and ovarian cancer, helper T cells may be essential for initiating, sustaining and amplifying an anti-tumor response.. CTL induced by stimulation with Ag in the presence of co-stimulation with B7- may become exhausted by the interaction with B7- tumor cells. The presence of Ag specific $CD4^+$ T cells may provide the "self-help" iieeded to sustain CTL responses (22, 23). Thus Thl cells recognizing peptides derived from the processing of HER-2 may produce cytokines (IFN-γ, TNF-α/β) that are thought to provide help for CTL function. The same HER-2 enitones mav produce Th2 cytokines in association with other MHC-class II types.

The objectives of this study were to (a) determine whether HER-2 peptide recognition occurs in healthy donors and in ovarian cancer patients with advanced disease and (b) whether distinct HER-2 peptides differ in their ability to modulate the cytokine secretion potential of the T cells from the same donor. In this report we present evidence that T cells responsive to multiple epitopes on a self-protein, HER-2, exist *in vivo* in healthy donors as well as in ovarian cancer patients. These cells can be stimulated to proliferate and expand *in vitro* and can secrete Th1 and Th2 cytokines. The observed *in vitro* responses of normal T cells to multiple peptides derived from HER-2 cannot be attributed to mitogenic effects by a particular peptide since (a) different peptides elicited PBMC proliferation in different donors and (b) three peptides containing the same P1 anchor for the same MHC-class II molecules failed to induce proliferative responses with the same frequency as four other peptides. Our analysis revealed that PBMC from ovarian cancer patients after chemotherapy responded less frequently than PBMC from healthy donors to the same peptides.

The reasons for the reduced frequency of responses in PBMC from ovarian cancer patients are still unknown. One possibility, to be addressed in future studies, is whether the ability of T cells from patients to respond is affected by chemotherapy. Chemotherapy can eliminate proliferating reacting clones to HER-2 peptides. An alternative is that individuals susceptible to ovarian cancer may be less responsive to self-antigen. This hypothesis could be tested in future studies using HLA-typed ovarian cancer patients. At this moment there is unknown whether there is a

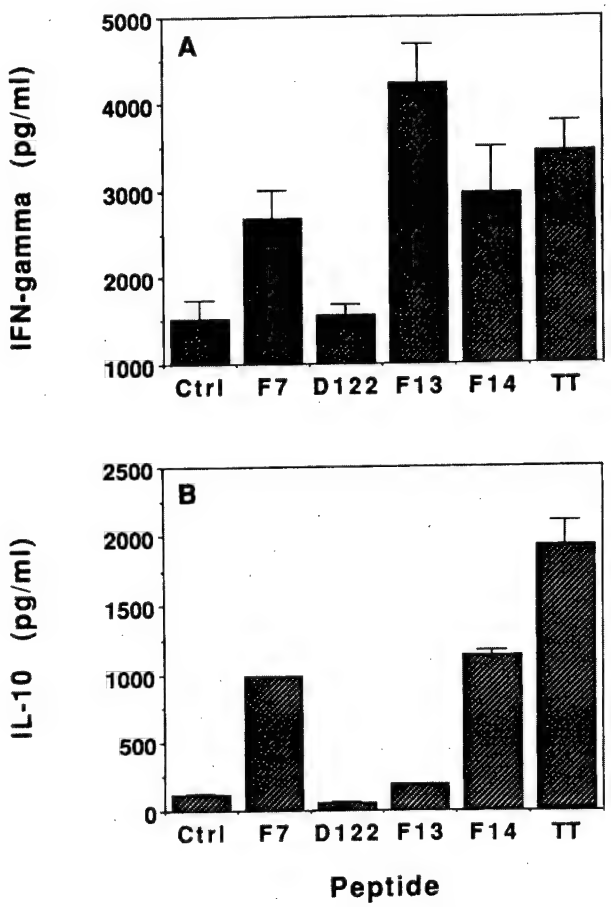


Figure 5. *Cytokine secretion by HER-2 peptides-induced T-cell lines. Equal numbers of cells (10^5) from each culture were plated in 96-well plates and incubated with PMA and OKT3 mAb as described in Materials and Methods. Cytokine expression was determined by ELISA. The concentrations were calculated by comparison with standard plots of IL-10 and IFN-γ in the same assays.*

disproportionate expression of MHC-class II allele in the cancer patients that may account for the different response pattern to the peptide antigens. Other possibilities currently under investigation are whether (a) $CD4^+$ T cells from these patients are anergic to these peptides or (b) their response is suppressed (1, 22). Suppressive effects due to disease progression may account for the lack of responses to these peptides at this stage. Although the group of patients with stable disease is relatively small to allow conclusive comparisions to be made, three of seven patients with stable disease responded to F13 while only two of eighteen patients with progressive disease (11%) responded to F13. In several cases when both PBMC and TAL were available from the same patient, we found that only PBMC responded to these peptides, suggesting the presence in situ of potentially negative regulatory mechanisms. Our preliminary results on the pattern of cytokine responses to F7 and F13 show that for a number of patients IL-10 was detectable at 48h in the

peptide induced cultures but the levels of IFN-γ were below the levels of detection of the assay (10 pg/ml). PHA stimulated PBMC from the same donors secreted both IFN-γ and IL-10. In other patients, only F7induced PBMC secreted TNF-α and/or IFN-γ (Melichar *et al* manuscript in preparation).

In our study, PBMC from ovarian cancer patients responded less frequently to F13 than did PBMC from healthy donors. The frequency of responses to F7 and F13 in healthy donors (54 and 62%, respectively) does not correlate with the frequency of expression of HLA-DR4 (25%) in the human population, suggesting that these peptides can be presented by other class II molecules.

Our current study also sought to elucidate the ability of HER-2-peptide stimulated T cells to expand and secrete cytokines. In this case we studies PBMC from a patient who had shown a stable response to several HER-2 peptides over a six month period. T cell lines of predominantly $CD4^+$ phenotype were readily expanded by restimulation with these peptides and low concentrations of IL-2. Interestingly, the resulting T cell lines differed in their proportions of $CD4^+$ and $CD8^+$ cells in their pattern of IFN-γ and IL- 10 secretion.

One possible explanation for these observed differences is that peptides F13 and F7 function as Thl and Th2 epitopes respectively in association with certain MHC-class II molecules. Both F7 and F13 contain a set of P1-P4-P6 anchors for HLA-DR4, though these sequences differ in charge at the P4 anchor: R (782) in F7 and E (892) in F13 correspond to the motifs for peptide binding sites to the DRBl*0402 and DRBl*0401/0404 alleles (12, 23). Phenotypic analysis of cells in the T cell lines stimulated by these peptides revealed a significantly larger population of $CD8^+$ cells in F13- than in F7- stimulated T cell lines from this donor. Furthermore, a T cell response to the epitope HER-2:783-797 mapped with the peptide SRLLGICLTSTVQ was detected in a breast cancer patient with high level of HER-2 auto-antibodies (1). F7, HER-2 (777-789) overlaps with HER-2:783797 in the area 777 - 783. The possibility that T cells stimulated by F7 can provide help for Ig synthesis deserves further consideration.

The fact that T cells from healthy donors and ovarian cancer patients respond to HER-2 peptides, indicates that tolerance to some of these self-epitope is not induced. Ongoing studies aim to determine whether T cells induced by these peptides recognize the HER-2 protein, the restriction element and the dominant epitopes for induction of a Th1 response. The implications of the observed responses in immunity to, or progression of ovarian cancer deserve further consideration as to whether the responses to these peptides correlate with HER-2 expression, stage and clinical outcome. Such studies are currently in progress in our laboratory. The results presented in this study should be useful for investigation of the mechanisms of Ag specific immunity, auto immunity, tolerance and design of epitope specific tumor vaccines.

References

1 Disis ML, Callenoff E, McLaughlin G, Murphy AE, Chen W, Groner B, Jeschke M, Lydon N, McGlynn E, Livingston RB, Moe R, and Cheever MA: Existent T-cell and antibody immunity to HER-2/neu protein in patients with breast cancer. Cancer Res *54:* 16-20, 1994.

2 Ioannides CG, Fisk B, Fan D, Biddison WA, Wharton JT, and O'Brian CA. Cytotoxic T cells isolated from ovarian malignant ascites recognize a peptides derived from the HER-2/neu proto-oncogene. Cell Immunol *151:* 225-234, 1993.

3 Yoshino I, Goedegeboure PS, Peoples GE, Parikh AS, DiMain JM, Lyerly HK, Gazdar AF, and Eberlein TJ: HER-2/neu derived peptides are shared antigens among human non-small cell lung cancer and ovarian cancer. Cancer Res *54:* 3387-3390. 1994.

4 Peoples GE, Goedegebuure PS, Smith R, Linehan DC, Yoshino I, and Eberlein TJ: Breast and ovarian cancer-specific cytotoxic T lymphocytes recognize the same HER2/neu-derived peptide. Proc.Natl.Acad.Sci, USA *92:* 432-436, 1995.

5 Fisk B, Blevins TL, Wharton JT, and Ioannides CG: Identification of an immunodominant peptide of HER-2/neu-proto-oncogene recognized by ovarian tumor specific CTL lines. J Exp Med *181:* 2709-2717, 1995.

6 Ioannides CG, Platsoucas CD, and Freedman RS: Immunological effects of tumor vaccines II. T cell responses directed against cellular antigens in the viral oncolysates. In Vivo *4:* 17-24.1990

7 Falk K, Rotzschke O, Stevanovic S, Jung G, and Rammensee H-G: Pool sequencing of natural HLA-DR, DQ, and DP ligands reveals detailed peptide motifs, constrainsts of processing, and general rules. Immunogenetics *39:* 230-242, 1994.

8 Kropshofer H, Max H, Muller CA, Hesse F, Stevanovic S, Jung G, and Kalbacher H: Self-peptide released from class II HLA-DR1 exhibits a hydrophobic two-residue contactmotif. J Exp Med *175:* 1799-1803, 1992.

9 Malcherek G, Falk K, Rotzschke O, Rammensee H-G, Stevanovic S, Gnau V, Jung G, and Melms A: Natural peptide ligand motifs of two HLA molecules associated with myastenia gravis. Int Immunol *5:* 1229- 1237, 1993.

10 Sette A, Sidney J, Oseroff C, del Guercio M-F, Southwood S, Arrheuius T, Powell MF, Colon SM, Graeta FCA, and Grey HG: HLA Drw4-binding motifs illustrate the biochemical basis of degeneracy and specificity in peptide-DR interactions. J Immunol *151:* 3136-3170, 1993.

11 Hammer J, Valsasnini P, Tolba K, Bolin D, Higelin J, Takacs B, and Sinigaglia F: Promiscuous and allele-specific anchors in HLA-DR binding peptides. Cell *74:* 197-203, 1993.

12 Hammer J, Gallazzi F, Bono E, Karr RW, Guenot J, Valsasnini P, Nagy ZA, and Sinigaglia F: Peptide binding specificity of HLA-DR4 molecules: Correlation with rheumatoid arthritis association. J Exp Med *181:* 1847-1855, 1995.

13 Sinigaglia F, and Hammer J: Motifs and supermotifs for MHC class II binding peptides. J Exp Med *181:* 449-451, 1995.

14 Marshall KW, Wilson KJ, Liang J, Woods A, Zaller D, and Rothbard JB: Prediction of peptide affinity to HLA-DRBl*0401. J Immunol *154:* 5927-5933, 1995.

15 Barker RN, and Elson CJ: Multiple self epitopes on the Rhesus polypeptides stimulate immunologically ignorant human T cells. Eur J Immunol *24:* 1578-1582, 1994.

16 Elson CJ, Barker RN, Thompson SJ, and Williams NA: Immunologically ignorant autoreactive T cells, epitope spreading and repertoire limitation. Immunol Today *16:* 71-76, 1995.

17 Barker E, Mackewicz CE and Levy JA: Effects of Thl and Th2 cytokines on $CD8^+$ cell response against human immunodeficiency virus: Implication for longterm survival. Proc Natl Acad Sci USA *92:* 11135-11139, 1995.

18 Tsuchida T, Parker KC, Turner RV, McFarland HF, Coligan JE, and Biddison WE: Autoreactive $CD8^+$ T-cell responses to human myelin. Proc. Natl Acad Sci USA *91:* 10859-10863, 1994.

19 Salgame P, Abrams JS, Clayberger C, Goldstein H, Convit J, Modlin RL, and Bloom BR: Differing lymophokine proflles of functional subsets of human CD4 and CD8 T cell clones. Science (Wash. DC) *254:* 279-282, 1991.

20 Matsuda M, Salazer F, Petersson M, Masucci G, Hansson J, Pisa P, Zhang, QJ, Masucci MG and Kiessling R: Interleukin 10 pretreatment protects target cells from tumor- and allele-specific cytotoxic T cells and downregulates HLA class I expression. J Exp Med *180:* 2371-2376, 1994.

21 Croft M, Certer L, Swain SL, and Dutton RW: Generation of polarized antigenspeciflc CD8 effector populations: reciprocal action of interleukin (IL)-4 and IL-12 in promoting type 2 versus type 1 cytokine profiles. J Exp Med *180:* 1715-1728, 1994.

22 Allison JP, Hurwitz AA, and Leach DR: Manipulation of costimulatory signals to enahnce antitumor T-cell responses. Curr Opin Immunol *7:* 682-686, 1995.

23 Wu T-C, Guarnieri FG, Staveley-O'Carroll KF, Viscidi RP, Levistsky HI, Hedrick L, Cho KR, August JT, and Pardoll DM. Engineering an intracellular pathway for major histocompatibility complex class II presentation of antigens. Proc Natl Acad Sci, USA *92:* 11671-11675, 1995.

24 Topalian SL, Rivoltini L, Mancini M, Markus NR, Robbins PF, Kawakami Y, and Rosenberg SA: Human $CD4^+$ T cells speciflcally recognize a shared melanomaassociated antigen encoded by the tyrosinase gene. Proc Natl Acad Sci USA *91:* 9461-9465, 1994.

25 Nanda NK and Sercarz EE: Induction of anti-self-immunity to cure cancer. Cell *82:* 13-17, 1995.

26 Wucherpfennig KW, and Strominger JL: Selective binding of self peptides to diseaseassociated major histocompatibility complex (MHC) molecules: a mechanism for MHC-linked susceptibility to human autoimmune diseases. J Exp Med *181:* 1597-1601, 1995.

27 Pfeiffler C, Stein J, Southwood S, Ketelaar H, Sette A, and Bottomly K: Altered peptide ligands can control CD4 T lymphocyte differentiation *in vivo.* J Exp Med *181:* 1569-1574, 1995.

28 Kumar V, Bheardwaj V, Soares L, Alexander J, Sette A, and Sercarz E: MHC binding affinity of an antigenic determinant is crucial for the differential secretion of IL4/5 or IFN γ by T cells. Proc Natl Acad Sci USA *92:* 9510-9514, 1995.

29 Wucherpfennig KW and Strominger JL: Molecular mimicry in T cell-mediated autoimmunity: viral peptides activate human T cell clones specific for myelin basic protein. Cell *80:* 695-705, 1995.

30 Silver RF, Wallis RS, and Ellner JJ: Mapping of T cell epitopes of the 30-kDa α antigen of *mycobacterium bovis* strain bacillus Calmette-Guerin in purified protein derivative (PPD)-positive individuals. J Immunol *154:* 4665-4674, 1995.

31 Clerici M, and Shearer GM. The Th1 - Th2 hypothesis of HIV infection: new insights. Immunology Today *15:* 575-581, 1994.

33 Sheen-Chen S-M, Chou F-F, Eng H-L, and Chen W-J: An evaluation of the progr.ostic signiflcance of HLA-DR expression in axillary-node-negative breast cancer. Surgery *116:* 510-515, 1994.

34 Iarygin LM, Malyshev VS, Polianskaia IS, Denisov LE, Zimin II, Alekseeva PL, and Khotchenknoa NV. The clinical significance of determining the HLA-DR4 antigen in patients with breast cancer. Voprosy Onkologii *37:* 796-799, 1991.

35 Casoli C, Zanelli P, Adorni A, Starcich BR, and Neri, T: Serological and molecular study on the HLA phenotype of female breast cancer patients. Eur. J. Cancer *30:* 1207- 1208, 1994.

36 Lee JE, Reveille JD, Ross MI, and Platsoucas CD: HLA-DQBl*0301 association with increased cutaneous melanoma risk. Int. J. Cancer *59:* 510-513, 1994.

37 Apple RJ, Erlich HA, Klitz W, Manos MM, Becker TM, Wheeler CM: HLA DR-D9 association with cervical carcinoma show papillomavirus-type speciflcity. Nature Genetics *6:* 157- 162, 1994.

38 Takahashi T, Chapman PB, Yang SY, Hara I, Vijayasandhi S, and Houghton AN: Reactivity of autologous $CD4^+$ T lymphocytes against human melanoma. J Immunol *154:* 772-779, 1995.

39 Topalian SL, Rivoltini L, Mancini M: Human CD4+ T cells specifically recognize a shared melanomas-associated antigen encoded by the tyrosinase gene. Proc. Natl. Acad. Sci. USA 91:9461-9465, 1994.

40 Becker JC, Brabletz T, Czemy C, Termeer C, and Brocker EB: Tumor escape mechanisms from immunosurveillance: induction of unresponsiveness in a specific MHC-restricted CD4+ human T cell clone by the autologous MHC class II+ melanoma. International Immunology 5: 1501- 1508, 1993.

41 Matsuo H, Batocchi A-P, Hawke S, Nicolle M, Jacobson L, Vincent A, Newsom-Davis J, and Willcox N: Peptide-selected T cell lines from mysastenia gravis patients and controls recognize epitopes that are not processed from whole acetylcholine receptor. J Immunol *155:* 3683-3692, 1995.

42 Ioannides CG, and Freedman RS: T cell responses to ovarian tumor vaccines. International Reviewsof Immunology *7:* 349-364, 1991.

43 Dong R-P, Kamikawaji N, Toida N, Fujita Y, Kimura A and Sasazuki T. Characterization of T cell epitopes restricted by HLA-DP9 in streptococcal M 12 protein. J Immunol *154:* 4536-4545, 1995.

Received July 25, 1996
Accepted September 10, 1996

Proliferative and Cytokine Responses to Class II HER-2/neu-associated Peptides in Breast Cancer Patients[1]

Todd M. Tuttle, Brett W. Anderson, William E. Thompson, Jeffrey E. Lee, Aysegul Sahin, Terry L. Smith, Kenneth H. Grabstein, J. Taylor Wharton, Constantin G. Ioannides,[2] and James L. Murray[2]

Departments of Surgical Oncology [T. M. T., W. E. T., J. E. L.], Gynecologic Oncology [B. W. A., J. T. W., C. G. I.], Breast Medical Oncology and Bioimmunotherapy [J. L. M.], Pathology [A. S.], and Biomathematics [T. L. S.], The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030, and Corixa Corporation, Seattle, Washington 98104 [K. H. G.]

ABSTRACT

Previous studies have characterized the reactivity of CD8+ CTLs with ovarian and breast cancer. There is little information about the antigens and epitopes recognized by CD4+ T cells in these patients. In this study, we analyzed the ability of T cells from peripheral blood mononuclear cells of breast cancer patients to recognize HER-2/neu (HER-2) peptides. We found that 13 of 18 patients responded by proliferation to at least one of the HER-2 peptides tested. Of these peptides, one designated G89 (HER-2: 777–789) was recognized by T cells from 10 patients. Seven of nine responding patients were HLA-DR4+, suggesting that this peptide is recognized preferentially in association with HLA-DR4. Analysis of the specificity and restriction of the cytokine responses to G89 by G89-stimulated T cells revealed that these cells secreted significantly higher levels of IFN-γ than interleukin 4 and interleukin 10, suggesting priming for a Th0-T helper 1 response. The same pattern of cytokine responses was observed to the intracellular domain of HER-2 protein, suggesting that G89-stimulated T cells recognized epitopes of the HER-2 protein in association with HLA-DR4. Because HLA-DR4 is present in 25% of humans, characterization of MHC class II-restricted epitopes inducing Th0-T helper 1 responses may provide a basis for the development of multivalent HER-2-based vaccines against breast and ovarian cancer.

INTRODUCTION

Studies in animal models have demonstrated a significant role for T lymphocytes in antitumor immunity and have shown that CD8+ and CD4+ cells can mediate tumor rejection (1, 2). In recent years, significant emphasis has been placed on identifying epitopes recognized by tumor-reactive CD8+ CTLs. A remarkable feature of these Ags[3] is that they are nonmutated self proteins (3). This raises the possibility that CD4+ cells recognizing epitopes on the same self proteins in the context of MHC I and MHC II may also be present in cancer patients (4). CD4+ cells may either express direct killing or play a regulatory role in the differentiation of other CD4+ cells and of tumor-reactive CTLs (5).

The known repertoire of tumor Ag recognized by CD4+ cells is limited. There is little information on the restriction elements operating in each Ag system and the nature of responses (Th1/Th2) induced by self peptides activating CD4+ cells. Topalian *et al.* (6, 7) have identified HLA-DR4.1 (HLA-DRB1*0401)-restricted tyrosinase peptides that stimulated Th1 cytokine secretion by CD4+ melanoma TILs. This ability was shown to be dependent on the binding affinity of the peptide to HLA-DR (7). Yoshino *et al.* (8) have shown that CD4+ TILs secrete Th1 cytokines when presented with heat shock proteins associated with HLA-DR, suggesting that they may recognize peptides complexed to such proteins (8). CD4+ CTLs were shown to recognize a shared HLA-DR15 melanoma-associated Ag (9). MHC class II-restricted Th1 cytokine secretion by long-term cultured CD4+ TILs has also been reported in breast cancer patients, suggesting a HLA-DR4-associated response (10). Finally, autologous tumor-specific CD4+ CTLs have also been demonstrated in sarcoma restricted by HLA-DR4 and HLA-DR15 (11).

These studies have shown that *in vitro* cultured CD4+ cells of TILs can recognize class II-associated Ag. There is little information on the ability of class II-associated peptides from these Ags to induce and restimulate a response in healthy donors and patients with cancer. Proliferative responses of PBMCs reflecting responses by T cells to mutated Ras protein and peptides carrying the same mutation have also been detected in pancreatic and colon cancer patients vaccinated with the corresponding peptide (12, 13). In contrast, responses to w.t. or

Received 12/17/97; revised 4/28/98; accepted 5/15/98.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported in part by DAMD Grant 17-97-I-7098 (to C. G. I.) and the Nellie Connally Breast Cancer Research Fund (to J. L. M.). Peptide synthesis was supported in part by Core Grant CA16672.

[2] To whom requests for reprints should be addressed, at Department of Gynecologic Oncology, Box 67 (C. G. I.), or Department of Bioimmunotherapy, Box 02 (J. L. M.), The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: (713) 792-4561.

[3] The abbreviations used are: Ag, antigen; PBMC, peripheral blood mononuclear cell; HER-2, HER-2/neu proto-oncogene; S.I., stimulation index; APC, antigen-presenting cells; HA, influenza hemagglutinin; w.t., wild type; ICD, HER-2 intracellular domain; IL, interleukin; TIL, tumor-infiltrating lymphocyte; Th1, Th0-T helper 1; P1, position 1; PHA, phytohemagglutinin; NO, no peptide.

mutated Ras have not been found in healthy donors (12). However, the immunity directed against mutated tumor proteins may be targeted to w.t. epitopes (14, 15). Breast cancer patients developed anti-p53 antibodies and T cells that proliferated *in vitro* in response to w.t. p53 only when mutated p53 accumulated in their tumors (15), suggesting that the enhanced presentation of w.t. p53 was leading to a Th2 response. Similarly, a breast cancer patient with HER-2 overexpression (HER-2^{hi}) and anti-HER-2 antibodies developed T cells that proliferated in response to both HER-2 protein and short HER-2 peptides (16).

These results suggest *in vivo* priming by enhanced presentation of self peptides due to HER-2 protein overexpression. There is little information on the ability of CD4+ cells from healthy donors and from cancer patients who do not overexpress HER-2 (HER-2^{lo}) to respond to HER-2 peptides. This is important because identification of CD4+ cells reacting with self peptides may allow not only identification of "protective" tumor Ag, but allow optimization of design of tumor vaccines, by incorporating a "self helper" peptide(s) that can amplify and spread a Th1 response when the disease progresses.

We recently found that healthy donors responded with higher frequency than ovarian cancer patients to a number of HER-2 peptides. We hypothesized that CD4+ T cells recognizing HER-2 are not deleted from the immune repertoire of healthy individuals (17). Because the patients in that study were not HLA typed and had advanced disease, we decided to investigate the ability of HER-2 peptides to induce proliferative responses in healthy patients with primary breast cancer of defined MHC class II type. We focused on two HER-2 peptides that induced the most frequent responses in our previous studies. We found that peptide G89 (HER-2, 777–789) induced responses with higher frequency (10 of 18, 56%) in this group and significantly higher in the HLA-DR4+ patients (7 of 9, 78%) than the other peptides tested. There was no difference in the pattern of cytokine responses between one patient who overexpressed HER-2 (HER-2^{hi}) and one healthy donor who did not overexpress HER-2 (HER-2^{hi}), suggesting that the ability of patients with localized breast cancer to respond to G89 it is not affected by HER-2 overexpression.

MATERIALS AND METHODS

Subjects. PBMCs were obtained from 18 breast cancer patients and 6 healthy volunteers (three DR4+ and three DR4−). All patients, with one exception, were clinically free of tumor at the time of study. Of the healthy volunteers, three were HLA-DR4+, and the others were HLA-DR4− (*i.e.*, MHC II phenotype was: donor 4, DR7, 11, DQ 2, 6; donor 5, DR13, 14; donor 6, DR11, 15, DQ6, 7). Eleven patients had pathology stage I disease, 5 had stage II, and 1 had stage III. One patient (patient 16) had no primary tumor yet was classified as having breast cancer because of the presence of a positive lymph node. All except one patient had undergone surgery and were free of disease at the time of study. One patient had recurrent disease. All patients except one had 0–3 positive lymph nodes. Tumor from only one patient had Black's nuclear grade III (advanced pathological characteristics); the remainder were grade I or II.

HLA Class II Molecular Oligotyping. Genomic DNA extracted from PBMCs as described (18–22) served as the substrate for amplification of a polymorphic locus-specific fragment of the HLA class II gene by PCR. For the *-DQB1* and *-DRB* loci, the flanking primers used were as follows: DRB-AMP-A, 5′CCCCACAGCACGTTTCTTG; DRB-AMP-B, 5′CCGCTGCACTGTGAAGCTCT; DQB-AMP-A, 5′CATGTGCTACTTCACCAACGG; and DQB-AMP-B, 5′CTGGTAGTTGTGTCTGCACAC.

Because of the large number of *HLA-DRB* alleles and the numerous shared sequences between different alleles, *HLA-DRB* typing was carried out in a stepwise manner. First, group-specific *HLA-DR* typing was performed using the primers DR-AMP-A and DR-AMP-B. Oligonucleotide typing of this PCR-amplified DNA allowed discrimination of *HLA-DR1, -DR2, -DR3/6, -DR4, -DR5 (-DRw11), -DR7, -DR8/12, -DR9, -DR10, -DR52a, -DR52b/c*, and *-Drw53*. Because there are numerous variants of *HLA-DR1, -DR2, -DR4, -DR5 (-DRw11), -DR6, -DR8/12*, and *-DR52b/c*, further discrimination of these subtypes required a second PCR using group-specific primers plus DRB-AMP-B. They include DRB-AMP-1 for the *HLA-DR1* group, DRB1-AMP-2 or DRB5-AMP-2 for the *HLA-DR2* group, DRB-AMP-3 for the *HLA-DR3, -DR5, -DR6, -DR8, -DR12* group, DRB-AMP-4 for the *HLA-DR4* group, and DRB-AMP-52 for the *HLA-DRB3* genes of the *HLA-DRw52* group. The sequences of the primers were as follows: DRB-AMP-1, 5′TTCTTGTGGCAGCTTAAGTT; DRB1-AMP-2, 5′TTCCTGTGGCAGCCTAAGAGG; DRB5-AMP-2, 5′CACGTTTCTTGCAGCAGGA; and DRB-AMP-4, 5′GTTTCTTGGAGCAGGTTAAC.

For *HLA-DRw52*-associated *-DRB1* genes (*HLA-DR3, -DR5, -DR6, -DR8*, and *-DR12*), the sequences of the primers were as follows: DRB-AMP-3 (5′CACGTTTCTTGGAGTACTCTAC), *HLA-DRw52*, and DRB-AMP-52 (5′CCCAGCACGTTTCTTGGAGCT).

PCR products separated by electrophoresis were blotted to Hybond N+ membranes (Amersham Pharmacia Biotech, Arlington Heights, IL) hybridized with [γ^{32}P]ATP-labeled allele-sequence-specific oligonucleotide probes. *HLA-DQB1* alleles were determined by hybridization with probes corresponding to variable sequences around positions 23, 26, 37, 45, 49, 57, and 70 of the *HLA-DQB1* outermost domain. "Broad" *HLA-DR* groups [*HLA-DR1, -DR2, -DR3/6, -DR4, -DR5 (11), -DR12, -DR7, -DR8, -DR9, -DR10, -DRB3*0101, -DRB3*0201-*0301, -DRB4*0101 (-DR53)*] were determined by hybridization with oligonucleotide probes corresponding to variable sequences around positions 10, 28, and 37 of the *HLA-DRB1* outermost domain. Subtypes of *HLA-DR1, -DR2, -DR3/5/6/8/12, -DR4*, and *-DRw52* were determined by hybridization of the respective group-amplified DNA to oligonucleotides corresponding to variable sequences around positions 28, 37, 57, 70, and 86 of the *HLA-DRB1* outermost domain.

HER-2 Peptide Selection. Peptides tested were selected if they contained the T-cell sites in HER-2 predicted by the computer program ANT.FIND.M, the general binding motif for human class MHC II Ag, and the anchors for a number of MHC class II Ags (HLA-DR1, -DR3, -DR4, -DR11, and -DQ7; Refs. 23–28), the sum of whose allelic frequencies covers 75–100% of Americans. The general peptide binding motif for various human MHC class II molecules consists of a P1 anchor, *i.e.*, an aromatic or large aliphatic residue in the first 3–5 amino acids

Table 1 HER-2 peptides used in this study

The Tyr and Trp italicized in position 3 or 4 may constitute P1 anchors. Similarly, the Val, Leu, and Met italicized in positions 4 and 5 may also constitute P1 anchors.

Peptide code	Position	Sequence															
HA	307–319		P	K	*Y*	*V*	K	Q	N	T	L	K	L	A	T		
F12	449–464	G	I	S	*W*	*L*	G	L	R	S	R	E	L	G	S	G	L
G88	450–462		I	S	*W*	*L*	G	L	R	S	R	E	L	G	S		
F14	474–487	T	V	P	*W*	D	Q	L	F	R	N	P	H	Q	A		
F7	776–788	G	S	P	*Y*	*V*	S	R	L	L	G	I	C	L			
G89	777–789		S	P	*Y*	*V*	S	R	L	L	G	I	C	L	T		
F13	884–899	V	P	I	K	*W*	*M*	A	L	E	S	I	L	R	R	R	F
G90	886–898			I	K	*W*	*M*	A	L	E	S	I	L	R	R	R	

close to the NH_2 terminus, and other major but less essential anchors at P4, P5, P7, and P9, counting from the P1 anchor (26–28). Because many peptides are capable of binding to many different MHC class II molecules because their sequences contain overlapping binding motifs for MHC class II molecules (27, 28), each of the peptides synthesized contained at least two of three anchors for each HLA-DR Ag and the main P1 anchors for most class II alleles (Table 1). In peptides G88, G89, and G90, positions P3 and P4 are occupied by hydrophobic, aromatic, and aliphatic residues, in that order, to facilitate peptide binding in different frames.

The binding motifs of synthetic peptides may differ from those of natural ligands because the latter incorporate processing constraints in addition to binding requirements. Thus, peptides were synthesized by following, when possible, the common motifs for all MHC class II molecules defined by pool sequencing of naturally processed peptides (25). In F7, F13, and F14, the sequence was extended to include Pro N-terminal to either the Tyr (the P1 anchor for HLA-DR1, DR3, DR4, and DQ7) or the Trp (reportedly as the P1 anchor for HLA-DR4 and DR11). For comparative studies of the responses associated with HLA-DR4, 13-mer analogues of F12, F7, and F13 (*i.e.*, G88, G89, and G90, respectively) were selected, using the anchor alignment matching the standard HLA-DR4/DR1 helper epitope, HA peptide (HA: 307–319) (Table 1). The predicted binding affinities of these peptides for HLA-DR4 (as IC_{50}) according to Rothbard's algorithm were as follows: HA, 35 nM; G88, 180 nM; G89, 987 nM; and G90, 219 nM (29). Peptides were prepared by the Synthetic Antigen Laboratory of the M. D. Anderson Cancer Center, using a solid-phase method as described previously (17, 30). Their identity was determined by amino acid analysis. Their purity was 93–97% as determined by HPLC. Peptides were dissolved in PBS, aliquoted at 2 mg/ml, and stored frozen at −20°C until use. The codes used to identify HER-2 peptides were assigned by the Synthetic Antigen Laboratory.

Recombinant ICD. The ICD (Lys^{676}–Val^{1255}) was cloned by PCR from c-erbB-2 cDNA (provided by Dr. Jacalyn Pierce, National Cancer Institute). The ICD was expressed in *Escherichia coli* using a pET vector with an amino-terminal His tag. Recombinant ICD was purified from inclusion bodies by a combination of Ni^{2+} affinity chromatography, size exclusion, and ion exchange chromatography. The purified ICD was greater than 95% pure as judged by PAGE and Western analyses (data not shown).

Stimulation and Propagation of T Cells. Freshly harvested PBMCs from breast cancer patients and healthy volunteers were isolated by Ficoll/Hypaque centrifugation. CD4+ and CD8+ cells were isolated from the plastic nonadherent fraction with magnetic beads. Cells were cultured at 1×10^6 cells/ml in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) with 10% pooled human AB serum and antibiotics in 2 ml in each well of a 24-well plate (complete RPMI medium). HER-2 peptides were added at a final concentration of 25 μg/ml. In other wells, PBMCs were stimulated with 25 μg/ml HA peptide, PHA at a final concentration of 1:100, or medium alone. After 6 days of stimulation with each peptide, cultures were expanded with IL-2 (Cetus) at 20 units/ml for the following week (17, 31). To induce Ag-specific T cells, the cells were then "rested" for 3–4 days by culture in the absence of IL-2. Then, the cells were stimulated at a 1:1 stimulator:responder ratio with irradiated (10,000 Rad) PBMCs and pulsed with individual peptides for at least 90 min at 37°C before addition to the cultures as described (17). For expansion, 4–5 days later, 20 units/ml IL-2 was added to the cultures for 7 additional days. Surface Ag expression was determined by fluorescence-activated cell sorting analysis using a FACScan (Becton Dickinson, Sunnyvale, CA) with a log amplifier as described (17).

Proliferation Assays. For proliferation assays, a 100-μl aliquot was removed from each well of the 24-well plate of primary cultures after 4–6 days, as described (16). Quadruplicate samples were cultured in a 96-well plate with 1 μCi of [^{3}H]Tdr in a final volume of 200 μl. The cells were harvested 16 h later, and the radioactivity was counted in a Beckman LS3501 liquid scintillation counter (16). A proliferative response was defined as positive when differences in cpm values between cultures that received peptides and cultures that did not receive peptides were significant by the unpaired Student's *t* test ($P < 0.05$).

Cytokine Production. The ability to secrete IFN-γ, IL-4, and IL-10 was determined by culturing the PBMCs with the corresponding peptides. Supernatants were collected at different times and stored frozen at −20°C. The cytokine concentrations were measured by double sandwich ELISA using the corresponding kits provided by BioSource International (Camariyo, CA). The cytokine assays were calibrated with human recombinant IFN-γ, IL-4, and IL-10 to detect each cytokine in the range of 15–1000 pg/ml. The following homozygous B cell lines were obtained from the American Society for Histocom-

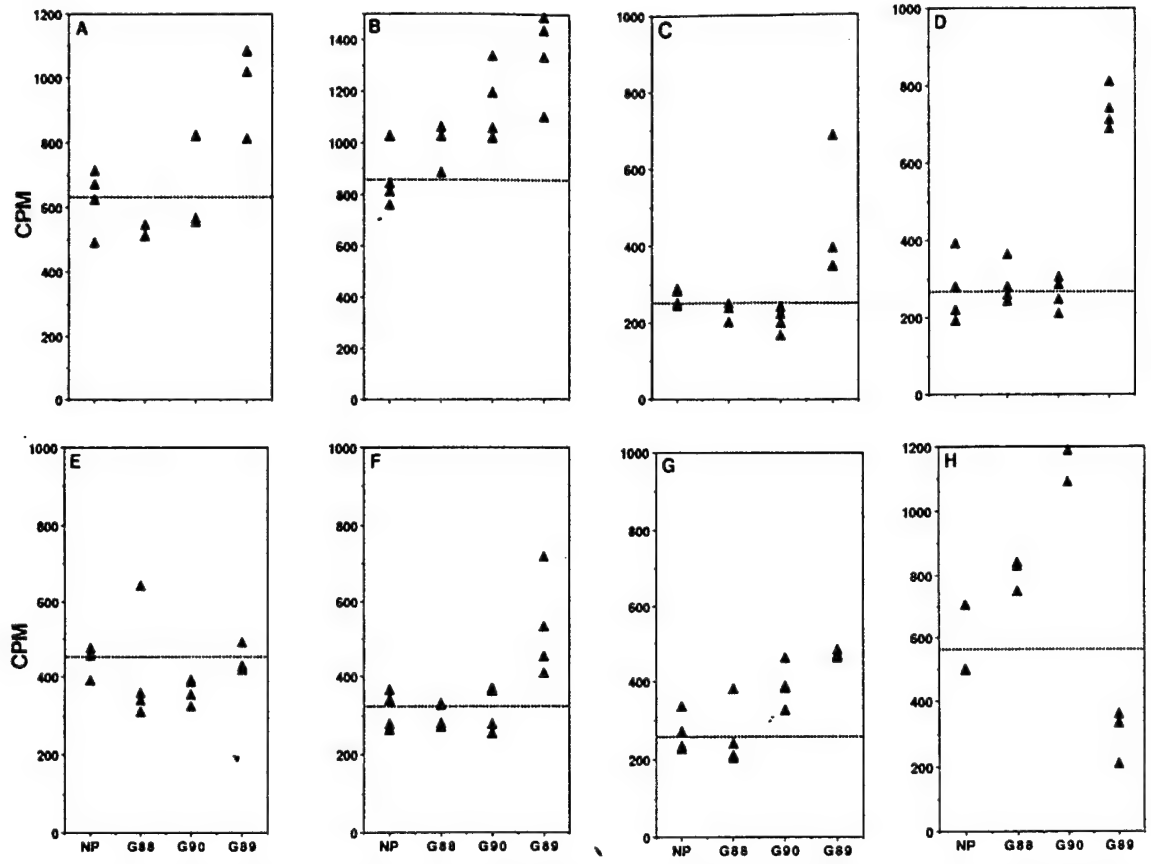


Fig. 1 Histograms of selected representative patterns of proliferation for eight breast cancer patients. *A*, patient 13, average NP value, 625 cpm; *B*, patient 7, average NP value, 862 cpm; *C*, patient 2, average NP value, 268 cpm; *D*, patient 9, average NP value, 272 cpm; *E*, patient 15, average NP value, 447 cpm; *F*, patient 12, average NP value, 313 cpm; *G*, patient 1, average NP value, 268 cpm; *H*, patient 5, average NP value, 568 cpm. Each determination was performed in quadruplicate; cpm for each of the replicates are represented by one triangle. Patients in *A–F* are HLA-DR4+ and those in *G* and *H* are HLA-DR4−. Responses to G89 were considered positive in *A–D* and *F* (HLA-DR4+ patients) and in *G* (HLA-DR4− patient) because in each of these donors tested, all cpm quadruplicate values in response to G89 were higher than each of the quadruplicate values of the control cultures unstimulated with peptide. Freshly isolated PBMCs from each donor were stimulated with peptides at a final concentration of 25 μg/ml. Responses were determined in 100-μl aliquots of cells removed from cultures on days 4–6 and tested for proliferation. Responses are shown for cultures stimulated for either 4 or 5 days. In most instances, significant proliferation was observed on two consecutive days (days 4 and 5 or days 5 and 6). An exception was made for patients 9 (*D*) and 15 (*E*), who showed responses only on days 5 and 6, respectively; patient 9 was considered a responder because the S.I. was >2.5, but patient 15 was considered a nonresponder because the S.I. to G89 was <2.0.

patibility and Immunogenetics Repository (Baltimore, MD) and used as APC for cytokine secretion: E418 (DRB1*1502, DRB5*0102, DQA1*0102, DQA1*0103, and DRB1*0601) and WT51 (DRB1*0401, DRB4*0101, DQA1*0301, and DQB1*0302).

Statistical Methods. Differences in proliferative responses were analyzed using Student's *t* test for unpaired samples. Differences in frequency for class II alleles were assessed using the Cochran Q test (32).

RESULTS

Recognition of HER2 Peptides in Breast Cancer Patients. PBMCs of breast cancer patients were cultured with HER-2 peptides or medium alone for 4–6 days. To ensure that lack of responsiveness of PBMCs to any of HER-2 peptides did not reflect a generalized suppression of responses to Ag or mitogen, all subjects' lymphocytes were tested for their ability to respond to PHA. Because responses to F7 (HER-2, 776–788) and F13 (HER-2, 884–899) were previously observed with higher frequency in healthy individuals (17), we wanted to address the question of the ability of the T cells from breast cancer patients to respond to HER-2 peptides, in association with certain MHC class II types, expression of HER-2 in their primary tumor, and the lymph node status. Because the patients were tested in the order they presented and not based on their HER-2 expression, when the S.I. was <2.0, to increase the sensitivity of detection, responses were considered positive when the cpm in each of the quadruplicate cultures stimulated with peptides was higher than each of the quadruplicate control cultures on two consecutive days (days 4 and 5 or days 5 and 6). This approach allowed us to identify responders without arbitrary cutoffs, using S.I.

Emphasis was given to HLA-DR4+ patients because of the recently reported association of HLA-DR and HLA-DR4 with

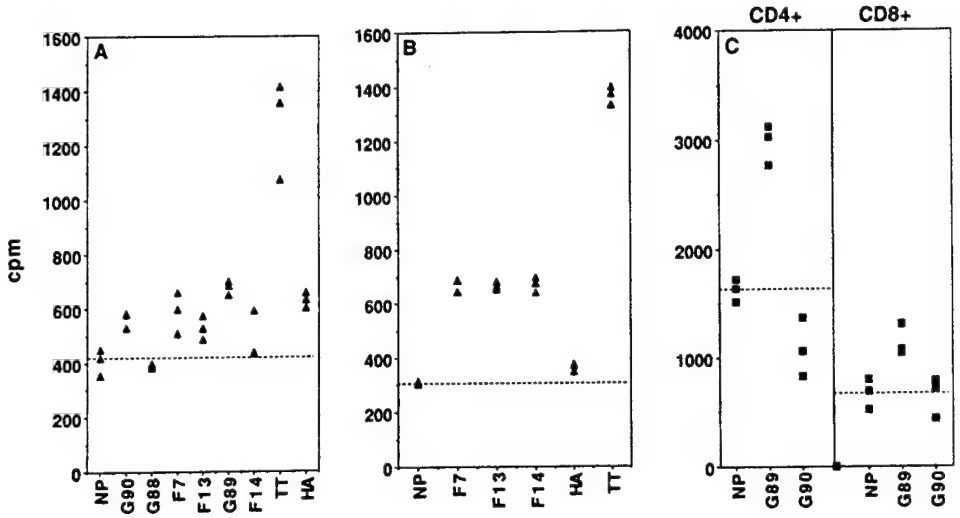


Fig. 2 Proliferative responses to HER-2 peptides by PBMCs from patient 13 (*A*; designated R; ▲), PBMCs from healthy donor 2 (*B*; ▲) determined in the same experiment with patient 13, and CD4+ and CD8+ cells from healthy donor 3 (*C*; designated L; ■). Both donors 2 and 3 were HLA-DR4+. The MHC class II phenotypes of the three healthy donors tested were as follows: donor 1, DR4, 13 (w52), DQ1, 3; donor 2, DR4, 15 w53, DQ6, 7; and donor 3, DR4, 13 w52, DQ1, 3. *A* and *B*, responses were determined 5 days after incubation with peptides in the same experiment for both donors. Responses in donor 2 were determined only to peptides F7, F13, and F14. Results similar to those of donor 2 were obtained for donor 1. *A* and *B*, *TT*, tetanous toxoid. See Table 2 for responses to HA peptide. *C*, responses were determined after 4 days of incubation with peptide, including the last 16 h the presence of ^{3}H-Tdr. Equal numbers of autologous plastic-adherent PBMCs were used as APC.

favorable prognosis in breast cancer (33, 34). Because both F7 and F13 contain HLA-DR4 anchors but differ in the length and position of the anchor motifs, we synthesized two 13-mers, designated G89 and G90. Each contained a hydrophobic aromatic followed by a hydrophobic aliphatic residue at P3 and P4 (Table 1). A control peptide of the same length: G88 (HER-2, 450–462) based on the sequence of F12 (HER-2, 449–464) was prepared (Table 1) because it had the same pattern of residues in P3 and P4 as G89 and G90. G88 was chosen as a control because responses to F12 were previously observed only infrequently (17). The predicted HLA-DR4 binding affinity of G88 was similar to that of G90 but significantly lower than that of G89.

The responses to HER-2 peptides G89 and G90, together with the responses to control peptide G88 for 8 of the 18 patients tested (including responders and nonresponders), are shown in Fig. 1. In all six G89 responding patients shown (Fig. 1, *A–D*, *F*, and *G*), the cpm values in all replicate cultures were higher than the cpm values in each replicate in PBMC cultures from the same patient that had not been stimulated with exogenously added peptide (NP). In these six patients, the cpm values in the replicate cultures stimulated with the control G88 peptide of higher binding affinity to HLA-DR4 than G89 were not significantly different from the cpm values in the NP cultures. This was confirmed by the fact than in the two nonresponders (Fig. 1, *E* and *H*), the cpm values in the replicate cultures stimulated with G89 were not higher than the cpm values in cultures stimulated with G88.

To verify the stimulatory ability of G89 in comparison with control peptide G88 and NP, the experiments were repeated with another patient (No. 13). Both peptides G89 and G90 and their counterparts F7 and F13 were tested in the same experiment. The results (Fig. 2*A*) show that responses to control peptide G88 were not significantly different from the NP cultures. Responses to G89 and G90 were significantly different from responses to NP or G89, but they were not significantly higher than responses to F7 and F13. The ability of PBMCs of HLA-DR4+ healthy volunteers to recognize HER-2 peptides was also tested. Significant proliferative responses by the same criteria were detected in HLA-DR4+ individuals after primary stimulation of PBMCs with various HER-2 peptides, of which responses to two donors (No. 2 and 3) are shown (Fig. 2, *B* and *C*). CD4+ cells responded to G89 (Fig. 2*C*). Thus, the ability to recognize sequences of the HER-2 protein is within the realm of the T-cell receptor of healthy volunteers, as we reported (17).

Proliferative responses to HER-2 peptides from all 18 patients tested are summarized in Table 2. G89 and F7 were recognized by PBMCs from 10 and 8 patients, respectively. PBMCs from six patients recognized both G89 and F7. Responses to G90 and F13 were observed in six and three patients, respectively. The results show a higher frequency of responses for G89 and G90 containing MHC class II anchors in P3 and P4 than for their analogues (F7 and F13) with the anchors shifted. The frequency of responses to G89 was significantly higher than to other peptides ($P = 0.02$). The results also show preferential association (7 of 9) of the responses to G89 with the presence of HLA-DR4 ($P = 0.01$). Of the other alleles that were represented, four of five HLA-DR3 patients responded to G89, but three of four responders were also HLA-DR4+. Four of six HLA-DR2+ patients responded to G89, but three of four responders were also HLA-DR4+. The other three HLA-DR4− donors responded preferentially to F13, with S.I. values of 1.9, 1.7, and 1.5, respectively, but no significant proliferation to G89 was observed (data not shown).

HER-2 staining for the autologous breast tumors was performed by immunocytochemistry. HER-2 was overexpressed

Table 2 Summary of proliferative responses of breast cancer patients to HER-2 peptides

Significant proliferative responses according to Student's *t* test are designated +. Responses not significantly different from those in control are designated −. All patients tested showed significant proliferation to PHA (data not shown). The allelism of the HLA-DQ has been determined and is listed. Values for control cultures that were not stimulated with peptides (NP) are listed as C.

Patient	DRB	DRB	DQB	DQB	NP	HA	G90	G88	PHA	F7	F13	G89	F14
1	3	11	301	201	C	−	+	−	+	+	+	+	+
2	4	7	301	201	C	−	−	−	+	+	−	+	−
3	2	4	303	602	C	−	−	−	+	−	−	−	−
4	2	7	201	502	C	−	+	−	+	+	+	+	+
5	6	7	303	303	C	+	+	+	+	+	−	−	+
6	1	7	303	501	C	−	−	−	+	−	−	−	−
7	2	4	302	602	C	+	±	−	+	+	+	+	+
8	3	4	201	201	C	−	−	+	+	−	−	+	−
9	2	4	302	602	C	−	−	−	+	+	−	+	+
10	2	2	602	602	C	−	−	−	+	−	−	−	−
11	3	4	301	501	C	+	−	−	+	+	−	+	−
12	2	4	301	602	C	−	−	−	+	−	−	+	−
13	3	4	302	604	C	+	+	−	+	−	−	+	−
14	3	6	402	501	C	−	−	−	+	−	−	−	−
15	4	7	301	301	C	−	−	−	+	−	−	−	−
16	6	11	603	604	C	−	+	−	+	−	−	−	−
17	6	6	N.D.	N.D.	C	−	−	−	+	−	−	−	−
18	8	8	301	501	C	−	−	−	+	−	−	+	−
Total						4	6	2	18	8	3	10	4
% positive						22.2	33.3	11.1	100	44.4	16.7	55.6	22.2

only in tumors from patients 6 (DR4−) and 13 and 15 (DR4+). The disease status, tumor size, lymph node status, tumor grade, and HER-2 expression were also compared with the response to these peptides. There was no correlation between proliferative responses and these clinical parameters.

No apparent correlation was found between HER-2 overexpression and proliferative responses to HER-2 peptides. Of the three HER-2^{hi} patients (two DR4+ and one DR4−), responses to G89 were seen only in patient 13 (DR4+). These responses were stable on both day 4 and day 5 of testing. In contrast, patient 15 responded only to F13 (on day 6) (Fig. 1*E*), whereas patient 6 (DR4−) responded only to G88 and F7. Of the seven HER-2^{lo} patients, five showed responses to G89 on at least two consecutive days of assay, one showed a response on day 5 (S.I. >2.5) (Fig. 1*D*), and one failed to respond. This group was too small for statistical analysis to address possible differences in response due to HER-2 overexpression and disease progression.

Analysis of the significance and specificity of responses focused on characterizing the proliferative and cytokine response to G89 by T cells from patient 13 (HER-2^{hi}). To address the specificity of proliferative responses, G89-primed PBMCs from patient 13 (Fig. 2*A*) were expanded in culture with IL-2 and retested for their proliferative responses to G88, G89, and G90. Freshly isolated monocytes/macrophages were not available from this patient for restimulation and follow-up studies. We used PHA blasts from the same patient as APC. Results in Fig. 3*A* show that G89-stimulated cultures (G89R) recognized G89 significantly better than G88 and G90, but the overall level of response was low. In fact, no clear improvement in proliferative activity was seen after expansion in IL-2 without restimulation with peptide. Because this may be due to the poor APC ability of PHA blasts, the specificity of G89 stimulation was characterized in parallel with the response from donor 3 (also HLA-DR4+) because autologous APC, plastic-adherent cells were available. Cultured G89-primed PBMCs of this donor (designated G89L) showed significantly higher proliferative responses to G89 than to control G88 peptide at restimulation

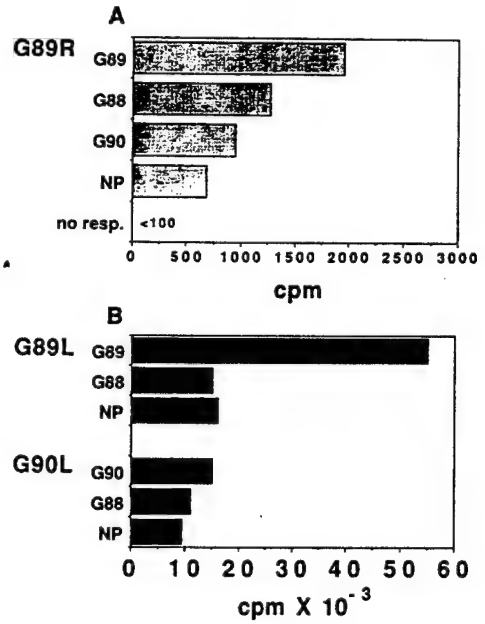


Fig. 3 *A*, specificity of proliferative responses of G89R T cells (derived after expansion in IL-2 of primary stimulated PBMC from patient 13). PHA blasts from patient 13 were used as autologous APC. *B*, specificity of proliferative responses of G89L and G90L (derived from donor 3). The G90L line was developed by priming with G90. Autologous plastic-adherent PBMCs were used as APC.

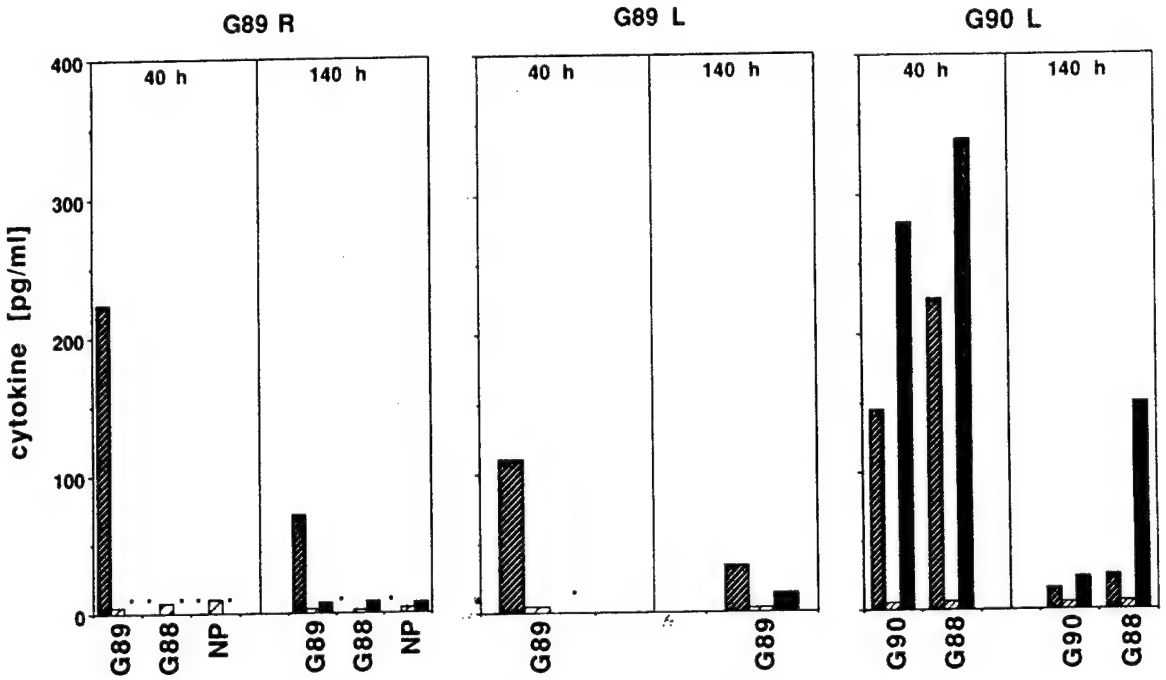


Fig. 4 Cytokine secretion by G89R and G90L T cells (5×10^4 cells each) in response to G89 and G90, respectively, pulsed on autologous irradiated PBMCs from donor 3 (1×10^5). G88 was used as control. Between 70 and 80% of cells had the CD4+ phenotype. Cytokine secretion by G89R and G90L was measured in the same experiment as described in "Materials and Methods." ▨, IFN-γ; ▨, IL-4; ■, IL-10. *, levels of this cytokine were below the sensitivity of the assay (2 pg/ml).

when presented on autologous plastic-adherent fraction of PBMCs (Fig. 3*B*). In contrast, the corresponding G90-primed (G90L cells) showed significantly lower specific proliferation to recall with G90 than G89L cells to recall with G89, suggesting that G90 may prime the T cells for a cross-reactive epitope.

Secretion of IFN-γ by G89-stimulated T-Cell Lines. Because the levels of IFN-γ and IL-4 in primary cultures were either very low or undetectable (data not shown), studies were conducted on secondary cultures. Recent studies have shown that IL-2 is required for Th2 differentiation and IL-4 production (35). To determine the type of cytokine responses to G89, cultures were established in low-dose IL-2 after initial stimulation of PBMCs with G89 from patient 13 (G89R) and donor 3 (G89L) and with G90 from donor 3 (G90L). The ability of these cells to secrete IFN-γ, IL-4, and IL-10 in response to the priming peptide was tested in parallel with the control peptide G88.

In preliminary experiments, we observed that the levels of IL-4 and IL-10 in response to G89 were low or undetectable. Because secretion of IL-4 and IL-10 may be delayed or HER-2 peptide G89 may be less efficient in inducing Th2 cytokines, we first determined the cytokine profile in response to G89 and G90 at both 40 and 140 h. The peptide G88 was used as control. The G89R T cells secreted high levels of IFN-γ in response to G89 but not in response to control G88 peptide (Fig. 4). These cells also secreted significantly less IL-4 and IL-10 than IFN-γ after either 40 or 140 h in culture, suggesting a preferential Th1 or Th0-Th1 response to G89. However, because the responses determined were obtained with short-term bulk cultures and because background levels of IL-4 and IL-10 were present, we defined this reactivity as Th0-Th1. The G90-primed G90L T cells showed significant cross-reactivity with G88 with regard to IFN-γ and IL-10 secretion and secreted significantly more IL-10 than did G89R G89-induced T cells. Although the levels of IFN-γ in response to wither G90 or G88 were higher than the levels of IL-4, the levels of IL-10 were higher than the levels of IFN-γ. Although it is possible that earlier levels of IFN-γ secreted in response to G90 may have been higher, the very high levels of IL-10 may suggest the presence of nonspecific Th2 cells activated following the initial G90 stimulation.

To address whether IFN-γ was secreted in response to G89 and the ICD (which contains this peptide but not the control G88 peptide), in association with HLA-DR4, G89R and G89L T cells were tested for cytokine secretion in response to G89 presented by PBMCs of different phenotypes (Table 3). Comparison of MHC I phenotype between APC and responders suggested that the IFN-γ was not secreted in response to MHC I. G89L shared HLA-A2 and HLA-B44 with APC from donor B. Although this may raise the possibility that G89, G88, or shorter fragments can be presented by HLA-A2, APC from donor C also shared HLA-A2 with G89L and expressed HLA-23, B41, and 81. However, the levels of IFN-γ secreted were low compared with the levels detected when G89 was presented by APC from donors A and B. Thus, although the possibility that MHC I molecules may present G89 cannot be excluded, comparison of the MHC I phenotypes suggest that G89-stimulated T cells secrete cytokines in response to MHC II molecules.

Significantly higher levels of IFN-γ than IL-4 were se-

Table 3 MHC class II restriction of peptide and HER-2 protein recognition by G89-induced T-cell lines

			G89L[a] (DR4, 15, DQ6, 7)		G89R[a] (DR4, 3, DQ3, 6)	
	APC	Peptide	IFN-γ	IL-4	IFN-γ	IL-4
A	A2, B7, 44	G89	250	10.5	341	39.5
	DR4, 15; DQ6, 7	G88	26	11.1	73	25.0
		ICD	298	17.9	662	10.0
	APC only	G89	<2	<2	<2	<2
B	A1, 2, B44, 57	G89	155	17.8	125	10.4
	DR10, 15, DQ1, 6	G88	<2	<2	<2	<2
		ICD	<2	<2	<2	<2
	APC only	G89	<2	4.7	<2	<2
C	A2, 23, B41, 81	G89	18.2	<2	59	<2
	DR7, 11, DQ2, 6	G88	2.2	2.5	<2	<2
			<2	<2	<2	<2

[a] The G89L and G89R T cell lines were stimulated with 1 μM HER-2 peptides (G89, G88) or 1 μM HER-2 ICD in the presence of autologous APC, with G89L, *i.e.*, APC sharing all DR and DQ with G89L, and HLA-DR4 and HLA-DQ6 with G89R (A); APC sharing only DR15 and DQ6 with G89L, and only DQ6 with G89R (B); and APC sharing only HLA-DQ6 with G89L (C). Supernatants were collected after 40 h in culture. Cytokine levels were determined as described in "Materials and Methods."

creted by G89L cells in response to G89 and ICD when G89 was presented by autologous APC to G89L. A similar pattern of response was observed for the G89R cells obtained from the breast cancer patient. The IFN-γ response by both G89L and G89R to the control G88 peptide, although significant, was at least one order of magnitude lower than to G89, or to the ICD. In this experiment, APC (from donor 3) and responders shared only HLA-DR4 and HLA-DQ6. Thus, in the presence of APC that shared either (*a*) DR15 and DQ6 with G89L, (*b*) DQ6 with G89L, or (*c*) DQ6 with G89R, significant levels of IFN-γ were observed in response to G89 but not to ICD, by both responders, the healthy donor and the cancer patient. The results also show that the restriction element used by G89L and G89R for recognition of exogenously added peptides is not exclusively HLA-DR4. These results suggest that it is likely that a naturally processed peptide from HER-2 is recognized by HER-2 peptide G89-primed T cells in the context of HLA-DR4.

IFN-γ and IL-4 release was observed in response to G89 (but not to ICD) presented by APC sharing either HLA-DR15 or HLA-DQ6 or both with the responders. These levels of cytokines were not induced in response to G88. Although the levels of cytokines secreted when G89 was presented by other HLA molecules were lower than levels in response to HLA-DR4, a certain pattern of "promiscuous" recognition was present, consisting always of higher levels of IFN-γ than IL-4. This suggests that although MHC class molecules of DR4-APC could present exogenously loaded G89 in a form recognizable to G89L and G89R T-cell receptor, the naturally processed and presented fragment of the ICD may have been derived from the one presented by DR4. A similar pattern of responses by G89L and G89R, although with reduced IFN-γ levels, was seen using lymphoblastoid cell lines WT51 (homozygous for DR4) and E4181324 (homozygous for DR15) as APC (data not shown).

DISCUSSION

In this report, we present evidence that PBMCs from primary breast cancer patients respond by proliferation *in vitro* to a number of HER-2 peptides. The responding population consists of CD4+ cells, as demonstrated in a previous study (16) and as suggested by the ability of the responding cells to secrete IFN-γ in response to these peptides when presented by MHC class II. In previous experiments, we noted that anti-MHC II antibodies and, to a lesser extent, anti-MHC I antibodies inhibited proliferation of PBMCs to helper peptides (17). Low levels of proliferation compared with CD4+ cells were observed with isolated CD8+ cells in a healthy donor, but the differences in cpm between G89-primed (G89L) and G90-primed CD8+ cells from donor 3 were significant. However, given the length of these peptides, the stimulatory potential for CD8+ cells after the binding of G89 to certain HLA class I allele products deserves further investigation. The frequency of the responses was higher for G89 (56%) than for the other peptides tested, suggesting that G89 may represent an immunodominant epitope in the group analyzed. Of interest, the responses to G89 appeared to associate more frequently with the presence of HLA-DR4 (in seven of nine cases), suggesting that HLA-DR4 may be the presenting element.

The fact that F7 and G89 are equal in length and differ by one residue at their NH_2- and COOH-terminal ends suggests that the epitope formed by G89 *in vitro*, when used at a concentration of ~10 μM, is specifically recognized. The frequency of responses appeared not to be related to the binding affinity of these peptides to DR4. The predicted binding affinity of G89 to HLA-DR4 was significantly lower than that of peptides G88 and G89 of the same length.

It is unknown at this time whether for G89, binding to HLA-DR is sequence specific, is restricted to certain DR4 subtypes, or is promiscuous. Depending on which P1 frame is used, Tyr or Trp can serve as an anchor for DRB1*0401 but not for *0404 and *0402. Similarly, at P4, negatively charged residues Asp and Glu are accepted by DRB1*0401 and *0404 but not by *0402, which accepts positively charged residues, such as Lys/Arg (36). This suggests that G89 (as well as G90) may preferentially bind to different DR4 subtypes and use alternative binding frames (*i.e.*, with Val, Leu, and Met for the P1 frame). Additional studies are required to address the ques-

tion of whether the antigenicity of G89 is associated with the predicted poor binding in a fashion similar to that reported for most tumor peptides from self Ag recognized by human CTLs.

T-cell cultures primed with G89 responded at restimulation by secreting more IFN-γ than IL-4 and IL-10, suggesting the preferential activation of a Th1 response. Because the experiments were performed with bulk cultures and not with clones, and because IL-4 and IL-10 were detectable, we would rather define this reactivity as Th0-Th1. This response was apparently not directed to a cryptic HER-2 epitope because peptide-primed cells recognized the ICD. The IFN-γ response to ICD of G89-primed T cells suggests that HLA-DR4 may be the presenting element for a naturally processed epitope similar in structure to G89.

Recent studies to examine proliferative responses in breast and ovarian cancer using HER-2 peptides of various lengths and randomly selected patients who had not been HLA-typed showed T-cell responses to several HER-2 peptides (16, 17). One of those, defined as p783 (HER-2, 783–797), reportedly activated responses of T cells to the HER-2 protein in a breast cancer patient (16). Although the magnitude of G89-induced responses was significantly lower than that reported for p783, our results indicate a trend of increased proliferation to G89. F7 (HER-2, 776–788) was also found to induce T-cell proliferation in both healthy donors and ovarian cancer patients (17). The data in this study suggest that within the area HER-2, 776–797, nests a dominant HER-2 epitope for CD4+ cells. Because HLA-DR4 is expressed in approximately 25% of humans, this epitope may be an important peptide for activation and regulation of T-cell differentiation toward a Th1 response. It may also be beneficial for CTL activation and expansion.

The observation that T cells from both healthy donors and patients whose tumors overexpress HER-2 can respond to G89 argues against the induction of tolerance to this epitope and/or against autoimmune activation of G89-specific T cells by HER-2 only after protein overexpression. In both this and the previous study with p783 (16), the proliferative responses were observed early, 4–6 days after stimulation. This may argue against a primary response to G89. Primary *in vitro* responses of T cells to some foreign Ag have been shown to require a significantly longer time (7–9 days) to be detected as significant proliferation (37, 38), although it is unknown whether these findings can be extended to self Ag. The low levels of cytokines at primary stimulation may even argue against a recall response, unless the frequency of G89-specific cells is very low. Additional studies are needed to clarify this point. A possibility that needs to be considered is that epitopes such as G89 may induce *in vivo* a limited number of Th1 cells, which may exert a regulatory function. Preliminary studies in our laboratory show that primary stimulation of T cells from healthy donors with either F7 or F12 or F13 followed by culture in IL-2 leads to preferential expansion of F7-responsive cells. These cells secreted high levels of IFN-γ at secondary and tertiary stimulation with F7. This pattern of responses suggests a determinant spreading effect as described for some cryptic epitopes (39).[4]

[4] B. W. Anderson, A. P. Kudelka, M. Pollack, J. L. Murray, and C. G. Ioannides,, manuscript in preparation.

Previous studies of HER-2 focused primarily on characterizing CTL epitopes (40, 41). The observation, in different systems, that human tumors are antigenic although poorly immunogenic emphasizes the need for development of approaches to induce and augment an immune response to tumor. Although *in vitro* and *in vivo* models show that induction of tumor-specific CTLs can be achieved by costimulation (42), the observed activation of Th1 response by the same tumor Ag recognized by CTLs suggests an involvement of CD4+ cells in the reaction to tumor. It also raises the question of whether the G89-induced Th0-Th1 response plays a protective role during tumor spread or whether it is down-regulated by Th2 cytokines subsequent to recognition of other peptides after HER-2 overexpression. In this context, the characterization of epitopes that regulate Th1 responses, which can in turn control the spread of Th1/Th2 responses by other self peptides, may have important implications not only for CTL induction but also for understanding the regulation of human tumor immunity.

ACKNOWLEDGMENTS

We thank Susan Mondragon for expert editorial assistance.

REFERENCES

1. Cheever, M. A, Britzmann-Thompson, D., Klarnet, J. P., and Greenberg, P. D. Antigen-driven long-term cultured T cells proliferate *in vivo*, distribute widely, mediate specific tumor therapy and persist long-term as functional memory T cells. J. Exp. Med., *163:* 1100–1112, 1986.

2. Rosenberg, S. A., Yannelli, J. R., Yang, J. C., Topalian, S. L., Schwartzentruber, D. J, Weber, J. S., Parkinson, D. R., Seipp, C. A., Einhorn, J. H., and White, D. E. Treatment of patients with metastatic melanoma with autologous tumor-infiltrating lymphocytes and interleukin 2. J. Natl. Cancer Inst., *86:* 1159–1166, 1994.

3. Boon, T., and van der Bruggen, P. Human tumor antigens recognized by T lymphocytes. J. Exp. Med., *183:* 725–729, 1996.

4. Mitchell, M. S. Attempts to optimize active specific immunotherapy for melanoma. Int. Rev. Immunol., *7:* 331–347, 1991.

5. Topalian, S L. MHC class II restricted tumor antigens and the role of CD4+ T cells in cancer immunotherapy. Curr. Opin. Immunol., *6:* 741–745, 1994.

6. Topalian, S. L., Rivoltini, L., Mancini, M., Markus, N. R., Robbins, P. F., Kawakami, Y., and Rosenberg, S. A. Human CD4+ T cells specifically recognize a shared melanoma-associated antigen encoded by the tyrosinase gene. Proc. Natl. Acad. Sci. USA, *91:* 9461–9465, 1994.

7. Topalian, S. L., Gonzales, M. J., Parkhurst, M., Li, Y. F., Southwood, S., Sette, A., Rosenberg, S. A., and Robbins, P. F. Melanoma-specific CD4+ T cells recognize nonmutated HLA-DR-restricted Tyrosinase epitopes. J. Exp. Med., *183:* 1965–1971, 1996.

8. Yoshino, I., Goedegebuure, P. S., Peoples, G. E., Lee, K-Y., and Eberlein, T. J Human tumor infiltrating CD4+ T cells react to B cell lines expressing heat shock protein 70. J. Immunol., *153:* 4149–4158, 1994.

9. Takahashi, T., Chapman, P. B., Yang, S. Y., Hara, I., Vijayasandhi, S., and Houghton, A. N. Reactivity of autologous CD4+ T lymphocytes against human melanoma. J. Immunol., *154:* 772–779, 1995.

10. Dadmarz, R., Sgagias, M K., Rosenberg, S A., and Schwartzentruber, D. J. CD4+ T lymphocytes infiltrating human breast cancer recognize autologous tumor in an MHC-class II restricted fashion. Cancer Immunol. Immunother., *40:* 1–9, 1995.

11. Heike, M., Schlaak, J., Schulze-Bergkamen, H., Heyl, S., Herr, W., Schmitt, U., Schneider, P. M., and Meyer zum Buschenfelde, K. H. Specificities and functions of CD4+ HLA- class II-restricted T cell clones against a human sarcoma: evidence for several recognized antigens. J. Immunol., *156:* 2205–2213, 1996.

12. Qin, H., Chen, W., Takahashi, M., Disis, M. L., Byrd, D. R., McCahill, L., Bertram, K. A., Fenton, R. G., Peace, D. J., and Cheever, M. A. CD4+ T-cell immunity to mutated ras protein in pancreatic and colon cancer patients. Cancer Res., *55:* 2984–2987, 1995.

13. Gjersen, M. K., Bakka, A., Breivik, J., Saeterdal, I., Solheim, B. G., Soreide, O., Thornsby, E., and Gaudernack, G. Vaccination with mutant ras peptides and induction of T-cell responsiveness in pancreatic carcinoma patients carrying the corresponding ras mutation. Lancet, *346:* 1399, 1995.

14. Takahashi, M., Chen, W., Byrd, D., Disis, M. L., Huseby, E., McCahill, L., Nelson, H., Peace, D., Qin, H. L., Shimada, H., Okuno, K., Yasutomi, M., and Cheever, M. A. Antibody responses to ras proteins in patients with colon cancer. Clin. Cancer Res., *1:* 1071–1077, 1995.

15. Tilkin, A. F., Lubin, R., Soussi, T., Lazar, V., Janin, N., Mathieu, M. C., Lefrere, I., Carlu, C., Roy, M., Kayibanda, M., Bellet, D., Guillet, J. G., and Bressac-dePaillerets, B. Primary proliferative T cell response to wild-type p53 protein in patients with breast cancer. Eur. J. Immunol., *25:* 1765–1769, 1995.

16. Disis, M. L., Calenoff, E., McLaughlin, G., Murphy, A. E., Chen, W., Groner, B., Jeschke, M., Lydon, N., McGlynn, E., Livingston, R. B., Moe, R., and Cheever, M. A. Existent T-cell and antibody immunity to HER-2/neu protein in patients with breast cancer. Cancer Res., *54:* 16–20, 1994.

17. Fisk, B., Hudson, J. M., Kavanagh, J., Wharton, J. T., Murray, J. L., Ioannides, C. G., and Kudelka, A. P. Existent proliferative responses of peripheral blood mononuclear cells from healthy donors and ovarian cancer patients to HER-2 peptides. Anticancer Res., *17:* 45–54, 1997.

18. Lee, J. E., Lowy, A. M., Thompson, W. A., Lu, M., Bell, B., Skibber, J. M., Evans, D. B., Curley, S. A., Mansfield, P. F., and Reveille, J. D. Association of gastric adenocarcinoma with the human leukocyte antigen class II gene. DQB1*0301. Gastroenterology, *111:* 426–432, 1996.

19. Lee, J. E., Reveille, J. D., Ross, M. I., and Platsoucas, C. D. *HLA-DQB1*0301* association with increased melanoma risk. Int. J. Cancer, *59:* 510–513, 1994.

20. Lee, J. E., Lu, M., Mansfield, P. F., Platsoucas, C. D., Reveille, J. D., and Ross, M. I. Malignant melanoma: relationship of the human leukocyte antigen class II gene *DQB1*0301* to disease recurrence in American Joint Committee on Cancer stage I or II. Cancer (Phila.), *78:* 758–763, 1996.

21. Lee, J. E., Loflin, P. T., Laud, P. R., Lu, M., Reveille, J. D., and Lawlor, D. A. The human leukocyte antigen *TAP2* gene defines the centromeric limit of melanoma susceptibility on chromosome 6p. Tissue Antigens, *47:* 117–121, 1996.

22. Marsh, S. G. E., and Bodmer, J. G. HLA class II region nucleotide sequences. Tissue Antigens, *45:* 258–280, 1995.

23. Kropshofer, H., Max, H., Muller, C. A., Hesse, F., Stevanovic, S., Jung, G., and Kalbacher, H. Self-peptide released from class II HLA-DR1 exhibits a hydrophobic two-residue contact motif. J. Exp. Med., *175:* 1799–1803, 1992.

24. Malcherek, G., Falk, K., Rotzschke, O., Rammensee, H-G., Stevanovic, S., Gnau, V., Jung, G., and Melms, A. Natural peptide ligand motifs of two HLA molecules associated with myasthenia gravis. Int. Immunol., *5:* 1229–1237, 1993.

25. Falk, K., Rotzschke, O., Stevanovic, S., Jung, G., and Rammensee, H-G. Pool sequencing of natural HLA-DR, DQ, and DP ligands reveals detailed peptide motifs, constraints of processing, and general rules. Immunogenetics, *39:* 230–242, 1994.

26. Wucherpfennig, K. W., and Strominger, J. L. Selective binding of self peptides to disease-associated major histocompatibility complex (MHC) molecules: a mechanism for MHC-linked susceptibility to human autoimmune diseases. J. Exp. Med., *181:* 1597–1601, 1995.

27. Hammer, J., Valsasnini, P., Tolba, K., Bolin, D., Higelin, J., Takacs, B., and Sinigaglia, F. Promiscuous and allele-specific anchors in HLA-DR binding peptides. Cell, *74:* 197–203, 1993.

28. Sinigaglia, F., and Hammer, J. Motifs and supermotifs for MHC class II binding peptides. J. Exp. Med., *181:* 449–451, 1995.

29. Marshall, K. W., Wilson, K. J., Liang, J. Woods, A., Zaller, D., and Rothbard, J. B. Prediction of peptide affinity to HLA-DRB1*0401. J. Immunol., *154:* 5927–5933, 1995.

30. Ioannides, C. G., Fisk, B., Fan, D., Biddison, W. A., Wharton, J. T., and O'Brian, C. A. Cytotoxic T cells isolated from ovarian malignant ascites recognize a peptides derived from the HER-2/neu proto-oncogene. Cell. Immunol., *151:* 225–234, 1993.

31. Ioannides, C. G., and Freedman, R. S. T cell responses to ovarian tumor vaccines. Int. Rev. Immunol., *7:* 349–364, 1991.

32. Fleiss, J. L. Statistical Methods for Rates and Proportions, pp. 83–85. John Wiley & Sons, New York, 1973.

33. Sheen-Chen, S-M., Chou, F-F., Eng, H-L., and Chen, W-J. An evaluation of the prognostic significance of HLA-DR expression in axillary-node-negative breast cancer. Surgery (St. Louis), *116:* 510–515, 1994.

34. Casoli, C., Zanelli, P., Adorni, A., Starcich, B. R., and Neri, T. Serological and molecular study on the HLA phenotype of female breast cancer patients (Letter). Eur. J. Cancer, *30:* 1207–1208, 1994.

35. Kamogawa, Y., Minasi, L. A., Carding, S. R., Bottomly, K., and Flavell, R. A. The relationship of IL-4- and IFN-γ-producing T cells studies by lineage ablation of IL-4 producing cells. Cell, *75:* 985–995, 1993.

36. Hammer. J., Gallazzi, F., Bono, E., Karr, R. W., Guenot, J., Valsasnini, P., Nagy, Z. A., and Sinigaglia, F. Peptide binding specificity of HLA-DR4 molecules: correlation with rheumatoid arthritis association. J. Exp. Med., *181:* 1847–1855, 1995.

37. Barker, R. N., and Elson, C. J. Multiple self epitopes on the Rhesus polypeptides stimulate immunologically ignorant human T cells. Eur. J. Immunol., *24:* 1578–1582, 1994.

38. Elson, C. J., Barker, R. N., Thompson, S. J., and Williams, N. A. Immunologically ignorant autoreactive T cells, epitope spreading and repertoire limitation. Immunol. Today, *16:* 71–76, 1995.

39. Nanda, N. K., and Sercarz, E. E. Induction of anti-self-immunity to cure cancer. Cell, *82:* 13–17, 1995.

40. Fisk, B., Blevins, T. L., Wharton, J. T., and Ioannides, C. G. Identification of an immunodominant peptide of HER-2/neu-proto-oncogene recognized by ovarian tumor specific CTL lines. J. Exp. Med., *181:* 2709–2717, 1995.

41. Peoples, G. E., Goedegebuure, P. S., Smith, R., Linehan, D. C., Yoshino, I., and Eberlein, T. J. Breast and ovarian cancer-specific cytotoxic T lymphocytes recognize the same HER-2/neu-derived peptide. Proc. Natl. Acad. Sci. USA, *92:* 432–436, 1995.

42. Allison, J. P., Hurwitz, A. A., and Leach, D. R. Manipulation of costimulatory signals to enhance antitumor T-cell responses. Curr. Opin. Immunol., *7:* 682–686, 1995.

antigens and separate adenocarcinoma antigens. Yamazaki, K., Savaraj, N., Spruill, G., Podack, E.R. *Department of Microbiology and Immunology, University of Miami, Miami, Fl 33101.*

In this study we wished to determine the CD8 positive, precursor CTL frequency for lung tumors, restricted by common HLA A alleles (A1, A2, A3). Cultured cell lines of small cell (SCLC) and adenocarcinoma of the lung were transfected with B7 plus A1, A2 or A3 and used as stimulators for purified CD8 cells from healthy volunteers partially matched at the A1, A2 or A3 locus. Primary culture of purified CD8 cells with partially MHC matched, B7 transfected tumor cells was carried out without feeder cells in the presence of low Il-2 and Il-4. Responder cell proliferation required weekly restimulation with tumor cells in the presence of feeder cells (irradiated PBL). A1 restricted CTL responses against SCLC were consistently found after 20 days in one donor but varied in other donors, suggesting variability in the responsiveness of different individuals. A1 restriction of CD8+ CTL was evident from increased cytolysis of A1 transfected SCLC and lower lysis of wild type SCLC or HLA transfected, but unmatched SCLC. A1 restricted, SCLC specific CD8+ CTL lysed A1 transfected SCLC tumor cells isolated from different patients, but not A1 transfected adenocarcinoma cells. A1 restricted precursor CTL frequency against SCLC was found at 5×10^{-4} and against adenocarcinoma at 5×10^{-5}. Our data indicate that direct antigen presentation can be used to measure precursor CTL frequencies. In addition our data suggest the presence of shared tumor antigens in SCLC and separate antigens in adenocarcinoma. These antigens may be useful for interventional immune strategies.

#569 Generation of human cytotoxic T lymphocytes recognizing wild-type sequence p53 epitopes from peripheral blood lymphocytes using autologous dendritic cells. Chikamatsu, K., Whiteside, T.L., Storkus, W., Lotze, M.T., and DeLeo, A.B. *University of Pittsburgh Cancer Institute, University of Pittsburgh, Pittsburgh PA 15213.*

The p53 gene is frequently mutated in human cancer, including squamous cell carcinoma. Therefore, overexpression of wild type and mutated determinants of the p53 protein may be useful as tumor antigens recognized by CTL for immunotherapy. In this study, we generated cytotoxic T lymphocytes (CTL) specific for p53 264–272 peptide from peripheral blood lymphocytes (PBL) of healthy donors by stimulation with autologous dendritic cells (DC), induced by GM-CSF/ and IL-4 and subsequent cultivation with cytokines (IL-1a, IL-2, IL-4, IL-6 and IL-7). The bulk CTL lyse HLA-A2+ cells pulsed with this peptide, but not HLA-A2+ p53 mutated tumor cells. Cloned CTL also do not recognize tumor cells. The CTL release GM-CSF, but not TNF. The results suggest that the affinity of these CTL may be too low to allow efficient recognition of tumors. Studies using DC pulsed with low concentrations of peptide, cultured with recombinant fragments of p53 protein or transduced with DNA encoding p53 exons to stimulate PBL are in progress. The analysis of anti-p53 CTL responses of HLA-A2+ patients undergoing p53 gene therapy is also underway to determine whether anti-p53 CTL occur at higher frequencies in their PBL. Our results confirm the existence of CTL recognizing wild-type sequence p53 epitopes, but further improvements are required to facilitate p53-based immunotherapy of cancer.

#570 Cytotoxic T cell responses for human carcinoembryonic antigen (CEA) epitopes in mice transgenic for HLA-A2.1K^b and human CEA. Mizobata, S., Clarke, P., Mann, J., Hefta, S.A., and Primus, F.J. *Wakayama Medical School, Wakayama 640, Japan, Beckman Research Institute of the City of Hope, Duarte, CA 91010, Vanderbilt University Medical Center, Nashville, TN 37232.*

The purpose of this study was to determine the immunogenicity in transgenic mice of two CEA peptides restricted by HLA-A2.1. One peptide, CEA-5 (VLYGPDTPII), was isolated from a CEA-transfected human breast carcinoma cell line (A-2.1$^+$). CEA-5 was structurally characterized by ESI-MS/MS that was facilitated by utilizing parent mass information obtained from the nontransfected cell line. CEA-5 was shown to bind to A2.1. The second peptide, CEA-4 (YLSGANLNL), was previously reported to elicit human CTLs in vitro (J. Natl. Cancer Inst., *87*:982, 1995). Both A2.1K^b and CEA/A2.1K^b transgenic mice were immunized with CEA-4 or -5 peptides in combination with helper peptide derived from hepatitis core protein. Using peptide-pulsed T2 or T2A2K^b targets, specific CTL activity against CEA-4 peptide was observed in A2.1K^b transgenic mice, but not in double transgenic mice. By contrast, CTL activity against the CEA-5 peptide was not observed in either transgenic mouse line. These studies show that the CEA-4 peptide is immunogenic in A2.1K^b transgenic mice while double transgenic mice are tolerant to this epitope. Alternative means of peptide immunization in double transgenic mice may break tolerance to CEA-4. Although the naturally processed CEA-5 peptide binds A2.1 well, it does not appear to be a CTL epitope in either A2.1K^b or CEA/A2.1K^b transgenic mice. These transgenic mouse lines will promote the identification of CEA-derived peptides restricted by HLA-A2.1 that may be potential CTL epitopes in humans.

antigens and is expressed by most melanomas. Immunization of mice aden virus (Ad) vector encoding gp100 has been shown by Zhai to in protective immunity against murine melanoma B16 challenge and efficiency presumed dependent on Ag presentation by the infected host APCs. This ob vation led us to test the strategy of targeting gp100 to dendritic cells (DC) *ex* and use Ag loaded DC as vaccine to enhance Ag specific immune response have previously demonstrated that bone marrow-derived DC can be re infected with Ad vector and 90% BM-DC were positive for β-gal 24 h follo infection with AdLacZ at moi of 100 pfu/cell. In the current study, immuniz s.c. with 2×10^8 DC transduced with Adgp100 resulted in 100% of mice b protected against a subsequent B16 challenge, whereas intramuscular inje with 10^9 pfu of Adgp100 vector resulted only in 10–20% protection. St cytotoxic T cell reactivities against B16 but not EL4 cells indicated that spe lytic CTL mediated DCAdgp100-induced protection. This antitumor activit volves both CD4- and CD8-positive T cells, in MHC class I- and II-restr responses, respectively. Investigation of effect of DC transduced with Adg on tumor-bearing animals is currently being carried out. (Supported by Canada)

#572 Murine dendritic cells transfected with gp100 elicit antigen cific, MHC restricted antitumor immunity. Yang, S., Darrow, T., Vervaer Seigler, H. *Duke Univ. Med. Center, Dept. Surgery, Durham, NC 27710*

DC are potent inducers of CTL when pulsed with antigenic peptide or t lysate. Here we have used liposome mediated gene transfer to investigate ability of TAA gene modified DC to elicit antitumor immunity. The gp100 mod DC (DCgp) were used to stimulate naïve nylon passed splenic T cells *in vitro* immunize mice *in vivo*. Antigen specific, MHC restricted CTL were gene when DC/gp were used to prime T cells both *in vitro* and *in vivo*. These CTL cytolytic for gp100 transfected syngeneic H-2^b tumor MCA106 (MCA/gp vac-pMel17/gp100 infected MCA and B16, but not parental tumor MCA or g modified H-2^d tumor P815. T-cell subset depletion experiments demonstrate induction of CTL *in vivo* is dependent on both CD4$^+$ and CD8$^+$ T-cells. The of DC transfected with plasmid containing a gene for TAA may provid alternative strategy for vaccine design.

#573 Cytolytic effector cell activity in peripheral blood from pancr cancer patients. Plate, J.M.D., Petersen, K.S., and Harris, J.E. *Rush-Pres rian St. Luke's Medical Center, Chicago, IL. 60612.*

The general immune status and tumor specific immunity were both studi pancreatic cancer patients. Peripheral blood mononuclear cells were obt from patients with metastatic pancreatic cancer who had not yet been tre with chemotherapy. The cells were examined for their ability to lyse diff pancreatic tumor cell lines. Cytolytic T cell activity was frequently obse against a pancreatic tumor cell line that expresses the tumor antigen M Some patients also exhibited non-T cell mediated cytolytic activity agains line as well as cytotoxicity against a second pancreatic tumor cell line that d express MUC1. These data indicated that a variety of immunologically rel markers are present on pancreatic tumor cells. Despite evidence of perip cytolytic effector cells, however, the tumors rapidly progress and kill the pat We hypothesized that the tumors themselves may defend against immune a by producing soluble inhibitors against cytolytic effectors cells. The hypot was tested by examining the effect of soluble MUC1 on cytolytic effecto activity *in vitro*. Soluble MUC1 readily inhibited T cell mediated cytotoxicity findings suggest that our patients' defense systems readily recognize panc tumor cells as foreign but their cytolytic effector cells are inhibited from era ing the tumor by an inhibitory concentration gradient encountered upon appr to the tumor site.

#574 Induction of Determinant Spreading in DR1101+ T cells Peptides Corresponding to HER-2 Helper Epitopes Results in a Th1 Cyt Response. Anderson, B.W., Pollack, M.S., Kudelka, A.P. and Ioan C.G. *M.D. Anderson Cancer Center, Houston, TX 77030.*

Determinant spreading involves recognition of additional autoantigenic d minants by T lymphocytes other than the priming Ag and a Th1/Th2 cyt response. We examined the ability of three peptides corresponding to H helper epitopes to induce determinant spreading. In two healthy DR1101 nors and one DR1101+ ovarian cancer patient, intramolecular spreading evident, as reflected by cell growth and Th1 cytokine responses produce PBMC which were stimulated by a different HER-2 peptide. High levels o gamma, but low levels of IL-4 and IL-10 were found in cultures stimulated and 3 times with alternative peptides. PBMC responded marginally at 1st ulation with F7(776–789), F12(449–464) and F13(884–899). At 2nd an stimulations, only cultures induced with F13 showed markedly higher resp to F7 and F12 than cells primed with the same peptide. This was not fou PBMC from a DR1101- healthy donor. F13 appears to be dominant, while F

candidate cryptic epitope in inducing a Th1 response. Intramolecular spreading may be used to develop an anti-tumor response in the HER-2 antigen system. Grant DAMD1797-I7098.

#575 CD4 T cells kill melanoma by mechanisms that are independent of Fas (CD95). Thomas, W., and Hersey, P. *Immunology and Oncology Unit, Division of Surgery, John Hunter Hospital, NSW, 2310. Australia.*

Previous studies have shown that CD4 T cells are associated with regression in primary melanoma and rejection of tumors in adoptive transfer models. The mechanism by which they mediate their antitumor effects remains unclear and in some studies it was suggested that Fas ligand (FasL)/Fas interactions were involved. In the present study we have examined the cytotoxic mechanism involved in CD4 T cell killing of melanoma cells and in particular the role of FasL/Fas interactions in this killing. We show that the CD4 T cells in four clones of T cells induced apoptosis in autologous melanoma cells by MHC restricted mechanisms but lysed an allogeneic melanoma cell by a non apoptotic mechanism. Melanoma cells were shown to express both Fas and FasL but killing of the melanoma cells did not involve Fas/FasL interactions. This was shown by lack of correlation between Fas expression and susceptibility to lysis and by failure of Mab to Fas to block killing by the CD4 T cells even though the latter expressed FasL. Recombinant FasL also did not induce killing of melanoma cells. The role of FasL on the melanoma cells and basis for the resistance of melanoma cells to FasL remains to be investigated.

#576 Autologous tumor cell cytotoxicity and cytokine biosynthesis in T cells infiltrating human lung cancers. Ortegel, J., Staren, E., Faber, P., Warren, W., and Braun, D. *Rush Medical College, Chicago, IL, 60612.*

The capacity of tumor-infiltrating T cells (TIL) from lung cancer specimens (n=14) to develop autologous tumor cytotoxicity (ATC) and express type 1 and type 2 cytokines was assessed following treatment with anti-CD3 antibodies + IL2 (CD3/IL2). ATC measured in 4 hr ^{51}Cr-release assays was 73.3±34 (lytic units/10^7 cells (LU) and 140.9±60 LU following culture in medium or CD3/IL2 respectively ($p<0.0005$). Induction of ATC was independent of tumor stage, histopathology, leukocytes/gm tumor and CD4:CD8 ratio. Intracellular type 1 cytokines (γ-IFN and TNFα) and type 2 cytokines (IL6 and IL10) were not detected by flow cytometry in TIL incubated in medium. However, within 6 hours of CD3/IL2 stimulation, CD8+ TIL made type 1 cytokines (15.3 ± 5% and 9.6±5% for γ-IFN and TNFα respectively) while CD4+ TIL made both type 1 and type 2 cytokines (7.2±5%, 12.6±4%, and 5.0±3% for γ-IFN, TNFα, and IL6 respectively). Addition of IL6 to TIL cultures stimulated with CD3/IL2 resulted in ≥ 50% reduction in ATC. The results show specific T cell activators induce ATC in TIL from human lung cancers but suggest that in vivo levels may be limited by induction of type 2 cytokines within the tumor microenvironment.

#577 Direct effect of tumor-derived factors in the downregulation of IFN-γ production by CD4+ T cells from tumor bearing mice. Cheng, X. and Lopez, D.M. *University of Miami School of Medicine, Miami, FL 33136.*

We have previously described that T cells from mammary tumor bearing mice have a downregulated production of IFN-γ. Although we have shown that tumor bearers have an impaired production of IL-12, we investigated whether tumor derived factors had a direct effect on IFN-γ production in normal T cells and its subsets. Pretreatment with GM-CSF resulted in increased IFN-γ levels by T cells, while PGE_2-pretreatment equally decreased the levels of this cytokine in CD4+ and CD8+ T cells from normal mice. Interestingly, phosphatidyl serine (PS) downregulated the IFN-γ production of CD4+, but not of CD8+ T cells. Methylation analysis indicated that CpG dinucleotide in SnaBI site of IFN-γ 5′ promoter flank region was hypermethylated in CD4+ but not in CD8+ T cells of large tumor bearers and of normal mice pretreated with PS. This correlated with their production of IFN-γ as measured by ELISA and RT-PCR. Electrophoresis mobility shift assay using an oligonucleotide probe corresponding to the IFN-γ promoter core region sequence showed a greatly reduced binding of a 90 kD nuclear protein in CD4+ T cells from tumor bearers and in those from PS-pretreated normal mice. Our data suggest that PS is involved in the IFN-γ gene downregulation in CD4+ T cells during mammary tumorigenesis.

#578 Impaired activation of NFκB in T cells from a subset of renal cell carcinomas (RCC) patients involves nuclear degrading of RelA. Ling, W., Rayman, P., Uzzo, R.G., Clark, P.E., Bloom, T., Kim, H.J., Hamilton, T., Tubbs, R., Novick, A., Bukowski, R., and Finke, J. *Cleveland Clinic Foundation, Cleveland Ohio 44195.*

Previously, we reported that supernatant fluid from explants of human renal cell carcinomas (RCC-S) but not normal kidney suppressed κB motif-specific binding activity in normal peripheral blood T lymphocytes without effecting the degradation of IκBα. Nuclear extracts from T cells cultured with RCC-S but not control cells caused degradation of RelA which was blocked by protease inhibitors. Here we provide evidence that in a subset of patients impaired NFκB activation may be attributable to degradation of Rel proteins following nuclear localization. In these patients (n = 10) the stimulus-dependent degradation of the inhibitor IκBα occurred normally. Time course studies demonstrated that in 6 of 10 cases κB binding activity was not detectable at any time examined. However, in 4 of 10 patients κB binding activity was detectable within 30 minutes but at reduced levels. In these patients DNA binding activity was transient and disappeared after 2 hours of stimulation. Immunoblotting, confirmed the transient nature of RelA, c-Rel and NFκB1 nuclear localization. Mixing experiments suggest that RelA is being degraded in the nucleus of patient T cells. Immunoblotting showed that RelA expressed in nuclear extracts of normal activated T cells was degraded when incubated with nuclear extract of patient T cells (resting or activated) which is devoid of RelA. Preliminary studies to characterize the product in RCC supernatant that can suppress NFκB activation demonstrate it is less that 3000 kDa and is sensitive to neuraminadase and partially sensitive to proteinase K. These findings suggest that a tumor product maybe the mediator of impaired NFκB activation in patient T cells through a mechanism that involves nuclear degradation of Rel proteins.

#579 Downregulation of the T cell receptor ζ chain in chronically stimulated Jurkat T cells. Garcia CS, Zea AH, Correa M and Ochoa AC. *Stanley S. Scott Cancer Center and Dept. of Pediatrics, Louisiana State University Medical Center, New Orleans, LA 70112.*

Upon antigen-induced T cell activation, the T cell receptor (TCR) $\alpha\beta$ heterodimer binds the peptide-MHC complexes while the ζ chain and the CD3 complex initiate the intracellular signal transduction cascade. The TCR then undergoes downmodulation and re-expression within 48 hours. Chronic stimulation of T cells also induces the cycle of internalization ans re-expression of the TCR but it is accompanied by a persistent decrease in the expression of the ζ chain which is evident as long as the stimulus is present. This treatment also results in anergy with a loss of the capacity of these cells to proliferate to further stimuli. We developed an *in vitro* model to study the mechanisms involved in the molecular alterations induced by chronic stimulation using Jurkat T cells stimulated with OKT3 antibody (1 μg/ml). Chronic stimulation of Jurkat with OKT3 resulted in a decreased expression of ζ chain which persisted for at least 8 days, while the expression of the CD3ϵ chain was similar to normal levels by day 8. Removal of the OKT3 resulted in the recovery of the ζ chain expression within 48 hours. Sequential immunoprecipitation with OKT3 and anti-ζ antibodies, respectively, suggest that the downmodulation of the ζ chain in chronically stimulated Jurkat T cells includes the ζ chain complexed to the TCR. Taken together, these data suggest that chronic stimulation of Jurkat cells can be a valuable *in vitro* model to study the alterations in signal transduction molecules seen in some cancer patients

#580 FasL-mediated monocyte-dependent CD4$^+$ T cells apoptosis in breast cancer patients following stem cell transplantation. James E. Talmadge, Rakesh K. Singh, Michelle L. Varney, Suleyman Buyukberber, Kazuhiko Ino, Ana G. Ageitos, Elizabeth Reed, Stefano Tarantolo. *University of Nebraska Medical Center, Omaha, NE 68198.*

The mechanism of peripheral tolerance in cancer patients has not been examined to date. We report the role of Fas-FasL interactions in monocyte-dependent T cell apoptosis by using a multicolor flow cytometric analysis including a TUNEL assay for apoptosis. Our data demonstrate that T cell apoptosis is significantly increased in the peripheral blood of breast cancer patients following high dose chemotherapy (HDT) and peripheral stem cell transplantation (PSCT). CD4$^+$ T cell apoptosis is significantly higher than that of CD8$^+$ T cells and is associated with an increased in Fas (CD95/APO-1) expression on these cells. The preferential depletion of CD4+T cells may contribute to the inversion of the CD4:CD8 ratio observed in patients following HDT and PSCT. A significantly increased frequency of monocytes and Fas-L expression on monocytes, parallels T cells apoptosis and suggests that the monocyte-associated FasL expression results in the selective depletion of CD4$^+$ T cells. We suggest that this is an important mechanism in the establishment of peripheral tolerance in breast cancer patients following HDT and PSCT.

#581 Ubiquitous intracellular Fas ligand expression by human and mouse tumor cells. Farhood, H., Hershberger, P., Shurin, G., Barksdale, E., and Lotze, M.T. *University of Pittsburgh, Pittsburgh, PA 15261.*

Fas ligand (FasL)-mediated induction of programmed cell death (apoptosis) in lymphocytes which express Fas receptor (Fas) is a natural mechanism for down regulation of the immune response. Evasion of an immune attack by expression of Fas ligand protein has been demonstrated for "immune privileged" organs such as the testis and eye. We screened a variety of human and mouse malignant tumor cell lines for FasL expression to test the hypothesis that tumor cells can express FasL and kill lymphocytes. Reverse-transcription of tumor cells RNA into complementary DNA (cDNA) followed by polymerase chain reaction (PCR) amplification of FasL cDNA demonstrated the presence of FasL mRNA in all human and mouse tumor cells tested. Expression of FasL protein in the same cells was ascertained by the Western blotting technique using tumor cell lysates. Every tumor cell tested was positive for high levels of FasL protein expression regardless of the tumor source. This includes renal cell carcinoma, lung carcinoma, hepatoblastoma, osteosarcoma, cervical carcinoma, lymphoma, melanoma, and neuroblastoma among others. The same cells were also shown to express abundant FasL protein using flow cytometry. However, flow cytometry results located the FasL protein in an intracellular compartment and not on the cell surface. This is contrasted to Fas expression by tumor cells which was readily detected extracellularly. Finally, tumor cells were able to kill a Fas-sensitive lymphocytic cell line in a JAM assay. All together, the data demonstrate an abundant FasL